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„Genetic, epigenetic and environmental modulators of the human stress response"

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"I am convinced that Natural Selection has been the main

But not exclusive means of modification."

Charles Darwin (1859)

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II Abbreviations

5-HIAA	Hydroxyindoleacetic acid
5-HTTLPR	Serotonin transporter gene-linked polymorphic region
ACh	Acetylcholine
ACTH	Adrenocorticotropin releasing hormone
AD	Anti depressant
ANS	Autonomic nervous system
ASPD	Antisocial personality disorder
AUC	Area under the curve
AVP	Arginine vasopressin
BDNF	Brain-derived nerve growth factor
BMI	Body mass index
CAR	Cortisol awakening response
CRH	Corticotropin releasing hormone
CSF	Cerebrospinal fluid
CTQ	Childhood trauma questionnaire
DEX	Dexamathasone
DNA	Deoxyribonucleic acid
DST	Dexamathasone suppression test
E	Epinephrine
EDTA	Ethylenediaminetetraacetic
ESLE	Early stressful life event
FA	Family adversity
G × E	Gene by environment
GC	Glucocorticoid
GR	Glucocorticoid receptor
GWAS	Genome wide association studies
HPA-axis	Hypothalamic-pituitary-adrenal axis
INDE	Insertion/Deletion
LSD	Lysergic acid diethylamide
MAOA	Monoamine oxidase A

MAO-I	Monoamine oxidase Inhibitor
MDD	Major depressive disorder
Mini-DIPS	Diagnostic interview for psychiatric disorder – short version
MR	Mineralocorticoid
NE	Norepinephrine
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFC	Prefrontal cortex
PSD	Post stroke depression
PTSD	Posttraumatic stress disorder
rh5-HTTLPR	Rhesus macaques 5-HTTLPR analogue
rs25531	5-HTTLPR/rs25531 mini haplotype
SCN	Suprachiasmatic nucleus
SNP	Single nucleotide polymorphism
SLE	Stressful life event
SPECT	Singe-photon emission computer tomography
TSST	Trier social stress test

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0 Abstract

Background In 2003, Caspi et al. reported a significant gene by environment (G × E) interaction effect between the short allele of the serotonin transporter gene-linked polymorphic region (5-HTTLPR) and early stressful life events (ESLE) on heightened depressive symptomatology. Ever since, the publications of contradictory findings and meta-analyses have fueled a vivid scientific debate on the existence of G × E interactions. One approach to reduce the complexity of interacting factors is the use of endophenotypes, such as the hypothalamic-pituitary-adrenal-axis' (HPA-axis) stress reactivity. The HPA-axis is influence by genetic, as well as environmental factors and various psychiatric disorders display a dysregulation of this system. Despite significant results from initial studies using endophenotypes in G × E research, the desired increase in explained variance could not be achieved. Leading scientists therefore suggest an even deeper immersion into the layers of biological information, as epigenetic profiles are able to directly influence gene transcription and are dynamically influenced by environmental signals. The present study therefore aims to investigate the influence of ESLE and the 5-HTTLPRgenotype on methylation levels in the promoter associated region of the serotonin transporter gene (*SLC6A4)*. Additionally, the role of methylation in this region in the context of the 5-HTTLPR on HPA-axis reactivity to psychosocial stress will be explored.

Method Healthy young (18 − 30y) adults (N = 186, 96 female) of Caucasian origin were genotyped for the 5-HTTLPR and 5-HTTLPR/rs25531 mini haplotype. To determine *SLC6A4* promoter-associated region mean methylation, DNA was extracted from whole blood, was then bisulfite-treated and the nucleotide sequence was determined by pyrosequencing. Furthermore, the history of ESLE (age < 13) was assessed using the Childhood Trauma Questionnaire (CTQ). The HPA-axis cortisol response to the Trier Social Stress Test (TSST) was determined using salivary cortisol samples.

Results A significant genetic with epigenetic interaction effect was identified between the 5-HTTLPRgenotype and mean methylation levels of 83 CpG sites in a 799 bp long CpG island, which has been previously defined by Philibert et al. (2008). The 'low' methylation group experienced a dose-dependent increase in cortisol secretion related to the S allele, explaining 7-9% of the observed variance. In contrast, the 'high' methylation group showed no differences in HPA-axis reactivity between genotype groups. Previously reported findings of an association between early traumatization and methylation profile in the *SLC6A4* promoter-associated region could not be confirmed.

Conclusion These findings highly support the importance of epigenetic data in psychiatric genetics and stress research. The inclusion of methylation data revealed an otherwise concealed effect and has therefore the potential to improve the current models. Until replication, these results remain preliminary.

1 Introduction

Mental disorders, which affect over 650 million people worldwide, are of pressing importance to all societies (Andlin-Sobocki, Jönsson, Wittchen, & Olesen, 2005; Lademann, Mertesacker, & Gebhardt, 2006). The most common of these diseases with 350 million incidences is major depressive disorder (MDD) (Marcus, Yasamy, van Ommeren, & Chisholm, 2012). Depression is among the leading causes of disability (Marcus et al., 2012) and is the number one contributor to years lived with disability (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006). Its clinical picture disrupts intrapersonal (emotion, cognition, behavior), interpersonal (family, friends), as well as professional life (work, school), and can end in suicide (Tamminga et al., 2002). Above the personal sphere, depression has huge social, socio-economical (health care for pharmaceutical and therapeutic treatment), and economical (lost work time, early retirement) costs (Greenberg et al., 2003; Simon, Ormel, VonKorff, & Barlow, 1995; Stewart, Ricci, Chee, Hahn, & Morganstein, 2003). Consequently the 65th World Health Assembly 2012 and the Mental Health Action Plan call for the identification of vulnerability and risk factors for MDD (World Health Organization, 2012b, 2013).

A crucial element in the etiology of mental disorders and especially MDD is stress, which led to the denomination of depression as a stress disorder (Nestler et al., 2002). Stress is an omnipresent experience in modern societies (Chrousos, 2009), but also in regions plagued by scarcity, poverty and war. Therefore stress is of central interest in the search for risk factors that compromise mental health. The two major paradigms in the 20th century that investigated this variable in the search of the origins of depression are the dichotomous traditions of environmental influences and genetic determinants, which are rooted in the dated 'nature vs. nurture' worldview. The environmental approach produced a voluminous literature (Dohrenwend, 2006) on the association between various SLE such as poverty, parental death, neglect or physical, sexual and emotional abuse, and depression (Agid, Kohn, & Lerer, 2000; Cicchetti & Toth, 2005; Grant, Compas, Thurm, McMahon, & Gipson, 2004; Hammen, 2005). Genetic research on the other side oriented itself on the preliminary theories of the working mechanisms of antidepressant (AD) drugs, which led to a focus on monoamine neurotransmitters, especially serotonin (5-HT) (Owens & Nemeroff, 1994).

Despite methodological advancements and positive findings in the respective fields, these separate models have not lived up to the initial expectations: The follow up reactions to SLE are heterogenic (Hoven et al., 2005; Monroe, 2008). Although after decades of research an association between stress and depression is established, many individuals do not develop psychopathological symptoms even after severe events (Monroe, 2008). Furthermore, the effects of single genes only explain a small degree of variance in complex psychiatric disorders (Levinson, 2006). Despite the incipiently promising results investigating the 5-HT 'master controller' (Nakamura, Ueno, Sano, & Tanabe, 2000), the serotonin transporter protein (SERT) and its corresponding gene (*SLC6A4*), subsequent research yielded inconsistent results and small effect sizes (Clarke, Flint, Attwood, & Munafò, 2010).

A recent approach to resolve these problems is the investigation of biological substructures that lye between the transcription of the genetic code to the emergence of self-awareness and psychopathologies. Such an intermediary 'endophenotype' is the hypothalamic-pituitary-adrenal axis (HPA-axis). Together with the autonomous nervous system (ANS), the HPA-axis constitutes the human stress (response) system (Pinel & Pauli, 2012). It has been intensively investigated concerning changes to its functioning through stress (Chrousos, 2009), genes (Mormède et al., 2002), and depression (Chrousos, 2009). Interestingly, stressful occurrences during development have been found to 'program' the HPA-axis reactivity to future threatening situations (Heim, Newport, Mletzko, Miller, & Nemeroff, 2008). Furthermore has the HPA-axis activation pattern a heritable component, which in part depends on polymorphisms in the *SLC6A4* (Miller, Wankerl, Stalder, Kirschbaum, & Alexander, 2012). Finally, a dysregulation of this system can often be found in the depressed population (Chrousos, 2009).

Despite significant results in the respective fields that investigated the HPA-axis, the examination of these variables separately does not seem to lead to an understanding in the etiology of MDD. Therefore a new school of thought is transgressing these separated paradigms: the quickly emerging field of gene by environment (G × E) interaction research that acknowledges the interdependence of (genetic) predisposition and (environmental) context (Caspi & Moffitt, 2006). To observe the brain without its related framework: the circulatory system, the digestive tract, or the organisms' biosphere makes little sense. Likewise is the genomic code dependent on an environment to unfold in and form an organism that in turn can process the external world and adapt. The logical custom to apply an analytical division to an interdependent whole is similar to asking who or what produces the tone: the musician or the instrument (Pinel & Pauli, 2012).

These ideas gained momentum with the publication of two groundbreaking studies by Caspi et al. (2002, 2003) in 2002 and 2003. The later was able to show that a functional length polymorphism in the promoter-associated region of *SLC6A4* (5-HTTLPR) was linked with depressive outcome, but only when additionally SLE had been encountered. These paradigm-shifting results (Risch et al., 2009) of a genetically transmitted vulnerability to environmental stimuli inspired subsequent research into known candidate genes for psychiatric disorders, now in the context of environmental pathogens. After the first decade, conflicting results of the first meta-analysis have resulted in an ongoing intense scientific debate about the existence of G × E interactions (Wankerl, Wüst, & Otte, 2010). In part, these inconsistences can be attributed to the methodological heterogeneity, but also to a lack of understanding of the molecular operations responsible for the observed effects.

It is thus suggested by leading scientists of the field to incorporate the layers of epigenetic information in our current models (Champagne, 2008; Foley et al., 2009; Heim & Binder, 2012; Homberg & Lesch, 2011; Lester et al., 2011; Meaney, 2010; Yehuda et al., 2010). These chemical alterations are able to functionally alter the deoxyribonucleic acid (DNA) in the absence of changes to the nucleotide sequence itself. Furthermore, they seem partially governed by environmental cues (Foley et al., 2009) and are also affected by the subjacent DNA sequence (Hellman & Chess, 2010). Consequently epigenetic alterations are a promising candidate to be a molecular mechanism conveying G × E interactions.

The ambition of this thesis is on the one hand to investigate the influence ESLE and the 5-HTTLPRgenotype on DNA methylation in a 799 bp island in the promoter associated region of the *SLC6A4* as previously defined by Philibert et al. (2008). On the other hand, the potential (interaction) effect of these epigenetic differences and the known factors ESLE and 5-HTTLPRgenotype on HPA-axis reactivity to psychosocial stress will be examined. This project is in accordance with the initially stated request of the World Health Organization to investigate vulnerability and risk factors for mental health and also aims to expand our understanding of epigenetics and their potential role in G × E interactions and psychopathology. On a broader scale, this could contribute research into response variability in AD treatment (Huezo-Diaz et al., 2009) and the development of personalized therapy approaches (Holsboer, 2008; Mehta et al., 2013; Philibert et al., 2008). To achieve this endeavor, the information in human and animal models of various research fields such as, psychology, psychiatry, biology, neuroscience, psycho-neuroendocrinology, genetics, epigenetics, and psychopharmacology are integrated in an inclusive 'behavioral epigenetic' framework (Lester et al., 2011).

2 Theoretical Background

> „Men are not disturbed by things
>
> But by the views they take on them"
>
> *Epictetus*

2.1 Stress

The term stress (from lat. *strictus* 'tight, compressed, drawn together'), first adapted from physics into the social sciences by Hans Selye and Walter B. Cannon (Cannon, 1928; Contrada & Baum, 2011; Selye, 1936), describes a natural response of the organism to internal or external stimuli (Hellhammer & Hellhammer, 2008) that actually or anticipatorily threaten its homeostasis, which is its vital state of dynamic equilibrium (de Kloet, Joëls, & Holsboer, 2005; Lupien, McEwen, Gunnar, & Heim, 2009). These so called 'stressors' can be physiological (e.g. blood loss, temperature, pain) or psychological (e.g. anxiety, fear, anger) (Ulrich-Lai & Herman, 2009). Before these stressors trigger adaptive physiological and behavioral changes, they pass through cognitive and affective appraisal processes that determine their valence, which is the potential ability to cope and generate survival strategies[1] (Contrada & Baum, 2011; Lazarus & Folkman, 1984). This definition is adapted to the requirements of the present study and confines the complexity of the term stress, though other concepts have been suggested (Chrousos, 2009; Koolhaas et al., 2011; Levine, 2005; Romero, Dickens, & Cyr, 2009).

The physiological stress response is primarily conveyed by two distinguishable but interrelated and complementary systems (Gunnar & Quevedo, 2007). On the one hand is the autonomic nervous system (ANS), which is able to immediately alter the physiological and mental state via the direct innervation of target organs through the release of epinephrine (E) and norepinephrine (NE) through its ergotropic sympathetic arm and acetylcholine (ACh) by

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[1] Notwithstanding non-cognitive processing, e.g. systemic stress responses to hemorrhage or stimulation by proinflammatory cytokines (Graeff, Guimarães, De Andrade, & Deakin, 1996; Ressler & Nemeroff, 2000)

the trophotropic parasympathetic arm (Ulrich-Lai & Herman, 2009). On the other hand is the relatively slower functioning (up to tens of minutes) HPA-axis, which exerts its diverse effects on almost every tissue (including the brain) through release of the glucocorticoid (GC) cortisol from the adrenal cortex into the bloodstream (de Kloet et al., 2005; Sapolsky, 2000a). The HPA-axis is of particular interest to psychological stress research due to its heavy innervation by limbic structures, which are central in the processing of psychogenic stressors (Csermely, Korcsmáros, & Sulyok, 2007; Lupien et al., 2009; Ulrich-Lai & Herman, 2009). Accordingly, a dysregulation of the HPA-axis is observed in several types of psychopathologies, such as MDD (Bao, Meynen, & Swaab, 2008; Chrousos, 2009), and is therefore discussed in detail.

2.1.1 The Hypothalamic-Pituitary-Adrenal Axis

The innervation of the paraventricular nucleus (PVN) of the hypothalamus (which integrates input from various sources) is at the start of a two-step hormonal activation mechanism of the HPA-axis (Ulrich-Lai & Herman, 2009). The PVN receives input from inter alia the brainstem (homeostatic state), circadian and metabolic information from other hypothalamic nuclei, serotonergic innervation from the median raphé nuclei (via 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2c} receptors), and especially important for psychosocial research: signals from the prefrontal cortex (PFC), limbic structures, such as the amygdala (excitatory) and the hippocampus (inhibitory) (Birbaumer & Schmidt, 2002; Glatz, Mössner, Heils, & Lesch, 2003; Heisler et al., 2007; Herman, Ostrander, Mueller, & Figueiredo, 2005; Mormède et al., 2002; Pinel & Pauli, 2012; Swanson, Sawchenko, & Lind, 1986; Ulrich-Lai & Herman, 2009). Upon activation, the PVN secretes the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal vessel system of the median eminence (Fig 1), which connects it to the pituitary gland (Gunnar & Quevedo, 2007; Pinel & Pauli, 2012). Subsequently, the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary into the bloodstream is stimulated (Birbaumer & Schmidt, 2002; Gunnar & Quevedo, 2007). Through the systemic blood flow, ACTH reaches the zona fasciculata (inner adrenal cortex), were it triggers the synthesis and release of GC hormones (de Kloet et al., 2005; Ulrich-Lai & Herman, 2009).

The primary GC in humans is cortisol (Pinel & Pauli, 2012). After secretion into the circulatory system, cortisol reaches cells in nearly every tissue of the body (including the brain)

via passive diffusion and influences transcription of target genes by binding to specific glucocorticoid- (GR) and mineralocorticoid receptors (MR) that act as ligand-activated transcription factors (Gunnar & Quevedo, 2007; Heitzer, Wolf, Sanchez, Witchel, & DeFranco, 2007; Sapolsky, 2000a). It is by occupation of these receptors in limbic forebrain structures, especially in the hippocampus, that the basal (high affinity MR, involved with appraisal and onset) and acute (low affinity GR, involved in termination and memory storage) activity of the HPA-axis is regulated and terminated. The majority of these inhibitory (hippocampus, PFC), but also excitatory (amygdala) pathways are integrated by the hypothalamus and the bed nucleus of the stria terminals before being relayed back to the PVN (de Kloet et al., 2005; Herman & Cullinan, 1997; Ulrich-Lai & Herman, 2009). On the whole, cortisol blood concentration increases after initiation and peaks after 15-30 minutes, declines thereafter and reaches base level after 60-90 minutes (de Kloet et al., 2005).

It is important to note that upon release cortisol is bound to 90%-95% to the high affinity protein cortisol binding globulin and others (e.g. albumin, erythrocytes), and only the free cortisol can become biologically active in the described way (Foley & Kirschbaum, 2010). The resulting effects on the organism are broad and complex, potentially permitting, stimulating or suppressing the stress response (Sapolsky, 2000a). In general, GCs are associated with metabolism (gluconeogenesis, release of stored energy, and inhibition of storage), oxygenation, immunity, inflammation, sleep, appetite, reproduction, cardiovascular tone, neural function, and behavior (de Kloet et al., 2005; Kyrou & Tsigos, 2009; Sapolsky, 2000a). Furthermore, GCs support at basal secretion levels the ANS stress response by permitting E and NE to fully effect their target organs (Gunnar & Quevedo, 2007; Sapolsky, 2000a). Contrarily during acute HPA-axis activation, GCs dampen the primary stress, immune, and inflammatory responses and protect the body from an excessive activation and overshooting of the ANS (de Kloet et al., 2005; Sapolsky, 2000a). The basal activity of the HPA-axis follows an 24 hour rhythmicity that is governed by the hypothalamic suprachiasmatic nucles (SCN) and consists of 20 brief, but intense bursts of secretion (Fries, Dettenborn, & Kirschbaum, 2009; Perreau-Lenz, Pévet, Buijs, & Kalsbeek, 2004; Van Praag, de Kloet, & Van Os, 2004). These episodes are not equally distributed in frequency and amplitude: instead concentration of cortisol increases during the second half of the night, peaks during the morning hours (before activity), declines thereafter and reaches nadir in the first half of the night (Fries et al., 2009). In addition, most people exhibit the cortisol awakening response (CAR) in the morning (Contrada & Baum, 2011; Fries et al., 2009). The CAR is characterized by a rapid increase of 38-78% in cortisol blood concentration levels 20-

45 minutes after awakening (Contrada & Baum, 2011; Fries et al., 2009; Schlotz, Hellhammer, Schulz, & Stone, 2004).
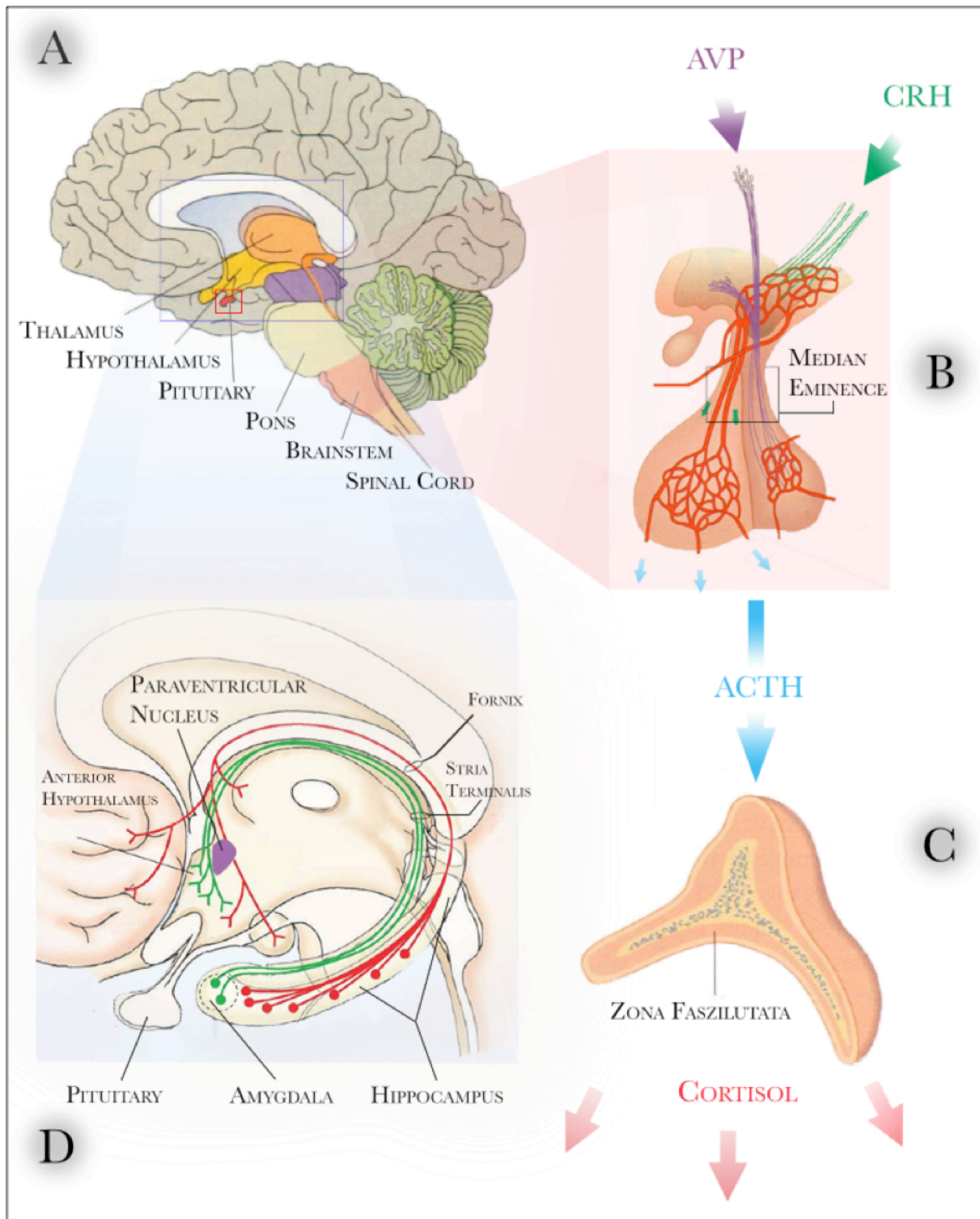
Figure 1 The hypothalamic-pituitary-adrenal axis (Birbaumer & Schmidt, 2002; Pinel & Pauli, 2012). **A**: Sagittal view – human brain **B**: Magnified pituitary gland. The transportation pathways of arginine vasopressin (AVP, purple) and corticotropin-releasing hormone (CRH, green) producing neurons of the paraventricular nucleus (PVN) are pictured. Further illustrated are the portal vessel system (red) and the median eminence (black). The arrows (blue) indicate the release of ACTH from the anterior (left) pituitary into the bloodstream. **C**: the adrenal gland (located on top of the kidneys). The Zona Faszilutata is the GC producing layer of the adrenal, which releases cortisol upon innervation by ACTH. **D**: magnified limbic system and important connections. Highlighted is the Fornix (red) connecting the hippocampus to the thalamus and hypothalamus; the Stria Terminalis (green) primarily linking amygdala and hypothalamus.

Independently of this general pattern, a variety of variables have been found to contribute to inter-individual differences in HPA-axis functioning: the presence of psychiatric disorders, such as MDD and posttraumatic stress disorder (PTSD) (overview see: Chrousos, 2009), somatic diseases, personality traits, social environment, gender, oral contraceptive, menstrual cycle, pregnancy, age, food intake, body mass index (BMI), alcohol, and nicotine (Chrousos, 2009; Foley & Kirschbaum, 2010; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999; Kirschbaum, Pirke, & Hellhammer, 1995; Kirschbaum et al., 1997; Nierop et al., 2006; Otte et al., 2005; Stalder et al., 2012). Moreover, genes (Miller, Wankerl, Stalder, Kirschbaum, & Alexander, 2012) and epigenetic markers (Ouellet-Morin et al., 2013) have been found to influence the HPA-axis. A moderate to high heritability of basal and stimulated HPA-axis activity has not only been reported in twin, family, and animal studies (de Kloet, Sibug, Helmerhorst, Schmidt, & Schmidt, 2005; Federenko, Nagamine, Hellhammer, Wadhwa, & Wüst, 2004; Foley & Kirschbaum, 2010; Mormède et al., 2002; Van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012), but also through a number of genetic polymorphism, such as variations in the *GR*, *MR*, and *SLC6A4* have been found to influence the HPA-axis response to stress (Foley & Kirschbaum, 2010; Miller et al., 2012; Way & Taylor, 2010).

Finally, converging evidence suggests environmental adversity during early developmental stages is able to persistently alter the HPA-axis response to stress (Anda et al., 2006; Gunnar & Quevedo, 2007; Heim & Nemeroff, 2001; Heim, Newport, Bonsall, Miller, & Nemeroff, 2001; Heim, Newport, et al., 2008; Heim, 2000; Heim et al., 2002; Heim, Plotsky, & Nemeroff, 2004; Kaufman, Plotsky, Nemeroff, & Charney, 2000; Lupien et al., 2009). Though a causal interpretation is problematic and conflicting results have been reported (Lovallo, Farag, Sorocco, Cohoon, & Vincent, 2012), this implication is strongly supported by a considerable body of literature on animal models (Levine, 2005; Lupien et al., 2009; Sánchez, Ladd, & Plotsky, 2001).

To investigate the HPA-axis reactivity, physiological (e.g. the cold pressor test, physiological strain), pharmacological (e.g. DST, DEX-CRH challenge[2]), and psychological

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[2] The dexamethasone (DEX) suppression test (DST) uses the characteristic of DEX to suppress the secretion of CRH and ACTH via feedback inhibition to identify (potential pathological) abnormalities in HPA-axis regulation. It was originally designed as a screening instrument for Cushing disease and later used to investigate HPA functioning in MDD and PTSD. The DEX-CRH challenge is a refined procedure, which observes the cortisol secretion in response to CRH administration after down regulation of the HPA-axis due to DEX the day before (Klaassen et al., 1999; Ruhé et al., 2007).

(e.g. public speaking, arithmetic tasks) stress tests can be administered (de Kloet et al., 2006; Foley & Kirschbaum, 2010). The most naturalistic and reliable standardized psychological laboratory test, is the Trier Social Stress Test (TSST) (Dickerson & Kemeny, 2004; Foley & Kirschbaum, 2010; Kirschbaum, Pirke, & Hellhammer, 1993; Kudielka, Hellhammer, & Wüst, 2009). This procedure combines the established stressors of public speaking, cognitive task, social evaluative threat, and uncontrollability, and has proven to induce a two- to threefold rise in cortisol concentration in 70% of subjects (Dickerson & Kemeny, 2004; overview see: Kudielka et al., 2009).

Beside cortisol, there are several other agents (e.g. CRH, ACTH) and attendant phenomena (e.g. heart rate, blood pressure) that indicate HPA-axis activity (Foley & Kirschbaum, 2010). However "the best characterized HPA-axis marker for the response to acute psychosocial stress is the release of cortisol" (Foley & Kirschbaum, 2010, p. 92). Cortisol can be quantified in the cerebrospinal fluid (CSF), urine, hair, blood, and saliva (Foley & Kirschbaum, 2010; Heim, 2000; Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009; Miller, Chen, & Zhou, 2007; Stalder & Kirschbaum, 2012). Specifically saliva comprises certain advantages in psychological stress research (Kirschbaum & Hellhammer, 1994). First, only the free (unbound) cortisol is measured, which is usually the best indicator for the HPA-axis response (Foley & Kirschbaum, 2010). Second, saliva samples are easily applied, thus allowing high frequency acquisition and are therefore preferable in the investigation of intraindividual variation over time (Kirschbaum & Hellhammer, 1994). Third, another advantage of their application is that they can be used in a variety of settings, are non invasive, versatile and avoid confounding through unintended stress provocation (Kirschbaum & Hellhammer, 1994).

To summarize, the evolutionary role of the HPA-axis is to provide energy in life-threatening situations, suppress non-relevant physiological processes, and facilitate and counterbalance the ANS stress response. It is of major interest in psychological stress research, due to its innervation by limbic and higher cortical sites that enable the integration and processing of emotions, appraisal, and reasoning. However, our conditions of living have changed dramatically from primarily immediate physiological threats to mainly psychogenic stressors, such as anticipated threat, social evaluation, shame, worrying, and expected outcomes. Interestingly these reactions originate from imaginary processes, leading to a physiological reaction geared towards survival in extreme situations, which are rarely present in modern societies. To no surprise, a dysregulation of this system has been observed in a

variety of psychiatric disorders. The development and the contribution of environmental, genetic, and epigenetic factors to an increased sensitivity to stressful stimuli are therefore a research aim of this thesis.

2.1.2 Environmental Strain – Stressful Live Event Research

The varieties of adverse experiences people encounter (such as loss, sexual, physical or emotional abuse, and natural disasters etc.) have been classified in various concepts. This study refers to Cohen, Kessler, & Gordon (1995), who define SLE as discrete, in principle observable and in time limited events that have sufficient impact to not be part of daily routine. In addition, they name the characteristics of undesirability, contextual threat, and lack of control (Cohen et al., 1995). In addition events occurring before the age of 13 are defined as early stressful life events (ESLE). This definition explicitly excludes other domains, such as chronic stress (McGonagle & Kessler, 1990; Miller et al., 2007), daily hassles (Kanner, Coyne, Schaefer, & Lazarus, 1981), and positive events (Cohen & Hoberman, 1983).

To quantify SLE, different methods have been employed (Paykel, 2001). *First*, self-report questionnaires, also called checklists, have became popular with the publication of Holmes & Rahes 'Social Readjustment Rating Scale' in 1967 (Dohrenwend, 2006; Paykel, 2001). Ever since, the use of checklists has become the by far widest used method, which has created an extensive literature with over 10.000 publications (Dohrenwend, 2006). Though substantial critique about memory and recall, reliability, validity, intracategory variability, confounding of measurement, and biased judgment has been raised since their introduction (Dohrenwend, 2000; Friis, Wittchen, Pfister, & Lieb, 2002; Hammen, 2005; Hobson & Delunas, 2001; Kendler, Karkowski, & Prescott, 1999; Kessler, 1997; Monroe, 2008; Paykel, 2001; Ungerer, Deter, Fikentscher, & Konzag, 2010), self-report questionnaires produce significant results, are economical and easy to administer, and are under ongoing development and improvement (Bifulco, Bernazzani, Moran, & Ball, 2000; Dohrenwend, 2006; Friis et al., 2002; Paykel, 2001). *Second*, structured and semi structured interview methods, which use complex rating and weighting schemes to quantify narrative interviews, try to incorporate the unique circumstances of the event (Dohrenwend, 2006; Monroe, 2008). Though several problems of the checklist approach are thereby addressed, interviews suffer themselves from drawbacks. These are amongst others the need of resource intensive schooling of the staff, the procedure of the interview itself, and the subsequent complex rating and weighting process (Dohrenwend, 2006; Kessler, 1997). *Third*, the acquisition of objective

data from official recordings in clinics, youth welfare or police has been suggested, but representative samples and willing participants are overall hard to obtain (Bernstein et al., 2003).

In conclusion, certain limitations in the endeavor of retrospectively conceptualizing subjective and unique experiences have to be accepted. This is also illustrated in the relative small to moderate variance SLE explain in psychopathology (Grant et al., 2004; Kessler, Price, & Wortman, 1985). Nevertheless, an association between stressful events and psychological symptoms has been consistently detected (see 2.1.2.1) (Grant et al., 2004) and many of the methodological problems concerning validity and reliability could been solved with the development of new improved questionnaires. One such instrument is the short version of the childhood trauma questionnaire (CTQ) (Bernstein et al., 2003). This easy to administer checklist has shown high internal consistency, reliability, and criterion validity and has been successfully applied in heterogeneous clinical and non clinical populations (Bernstein et al., 2003).

2.1.2.1 Stress in Mental Health

Under circumstances of inadequate and excessive activation, the vital stress response can become maladaptive and harmful to the organism (Bao et al., 2008; Chrousos, 2009; de Kloet et al., 2005; Gunnar & Quevedo, 2007; McEwen & Seeman, 1999; McEwen & Wingfield, 2003; McEwen, 2000, 2007). Epidemiological (Anda et al., 2006; Cicchetti & Toth, 2005; Friis et al., 2002; Mullen, Martin, Anderson, Romans, & Herbison, 1996), prospective longitudinal (Friis et al., 2002; Spataro, Mullen, Burgess, Wells, & Moss, 2004), clinical (Agid et al., 1999; Kessler et al., 1985), twin (Kendler, 2000), and single major event studies (Hoven et al., 2005), as well as comprehensive reviews of the literature (Agid et al., 2000; Dohrenwend, 2000; Grant et al., 2004; Hammen, 2005; Kessler, 1997; Margolin & Gordis, 2000; Monroe, 2008; Schneiderman, Ironson, & Siegel, 2005) heavily support an association between SLE, MDD, and other psychiatric diseases. In the next section there will be a brief overview of central arguments from the extensive literature on SLE and depression, which is followed up by an elaboration on the detrimental effects of early traumata.

2.1.2.2 Stress and Depression

"Depression is often described as a stress-related disorder" (Nestler et al., 2002, p. 14) and despite former doubt (Kessler et al., 1985; Kessler, 1997), accumulated evidence strongly supports a robust and causative association between SLE and MDD (Hammen, 2005). SLE are particularly frequent (80%) preceding episodes of MDD (Eley & Stevenson, 2000; Hammen, Davila, Brown, & Ellicott, 1992; Hammen, 2005; Kendler et al., 1999; Kendler, 2000; Kessler et al., 1985; Kessler, 1997; Mazure, 1998; Mundt, Reck, Backenstrass, Kronmüller, & Fiedler, 2000; Paykel, 2003) and have a high prevalence in the depressed population (Hammen, 2005; Kessler, 1997). They furthermore have been reported to have a dose-response relationship with depression (Hammen et al., 1992; Kessler, 1997), to influence remission, relapse, chronicity (Agid et al., 2000), and the course of treatment (Monroe, Kupfer, & Frank, 1992).

The consequences of ESLE appear to be particularly detrimental, as they increase the risk of a depressive episode later in life (Agid et al., 1999, 2000; Anda et al., 2006; Bifulco et al., 2000; Cicchetti & Toth, 2005; Friis et al., 2002; Gross & Hen, 2004; Hammen et al., 1992; Hammen, 2005; Heim & Binder, 2012; Heim et al., 2004; Kessler, 1997; Margolin & Gordis, 2000; Spataro et al., 2004). There exist evidence that individuals who experience adversity in youth display an increased vulnerability to the pathogenic effects of later encountered stressful events (Heim, Mletzko, Purselle, Musselman, & Nemeroff, 2008; Heim, Newport, et al., 2008). This effect is possibly conveyed by neural adaption to the encountered adverse environment (Gunnar & Quevedo, 2007; Heim, Newport, et al., 2008; Lupien et al., 2009). This programming could turn out to be maladaptive later in life and render the individual susceptible to develop mental illness. The characteristic of MDD to be an early onset and recurrent disease might be a manifestation of such processes (Costello et al., 2002; Hammen et al., 1992; Hammen, 2005; Nestler et al., 2002). These adaptations might manifest in changes in the workings of the HPA-axis (Bao et al., 2008; Lupien et al., 2009; Strüber, Strüber, & Roth, 2014). Hypercortisolaemia[3] (melancholic-, atypical-, season, and climacteric depression) and hypocortisolaemia[4] (MDD & PTSD) (Anda et al., 2006; Chrousos, 2009;

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[3] A state of elevated systemic cortisol

[4] A state of decreased systemic cortisol

Cicchetti, Rogosch, Sturge-Apple, & Toth, 2010; Strüber et al., 2014) are common phenomena in depressed patients (Bao et al., 2008). Another characteristic of this disorder is an increased (Heim & Nemeroff, 2001; Heim, Newport, et al., 2008; Heim, 2000; Lupien et al., 2009), respectively decreased (Burke, Davis, Otte, & Mohr, 2005; Lovallo et al., 2012; Lupien et al., 2009; Strüber et al., 2014) reaction of the HPA-axis to stress. These contrasting workings of the HPA-axis exemplify the heterogeneity of the concept of depression, the investigated populations (clinical, childhood trauma, age, depression severity), and methodology (time of day, biological indicator, stress evoking procedures) (Burke et al., 2005). In the case of early traumatization, alterations of neuronal networks that regulate the HPA-axis and lead to an increase of the cortisol stress response have been at the center of the work of Christine Heim (Heim & Nemeroff, 2001; Heim et al., 2001; Heim, Mletzko, et al., 2008; Heim, Newport, et al., 2008; Heim, 2000; Heim et al., 2002; Rao, Hammen, Ortiz, Chen, & Poland, 2008) and is of central interest in the development of this thesis. Further empirical support for this diatheses stress model stems from a variety of animal models (overview see: Lupien et al., 2009; Pryce et al., 2005; Strüber et al., 2014). Yet again it has to be noted that there exist contrasting results of a blunted cortisol reactivity as a consequence of childhood trauma (Strüber et al., 2014). The described inconsistences could be in part attributed to variable genetic predisposition (2.1.3.1, 2.1.3.3) and differentiated epigenetic imprinting early in life (2.2.2, 2.2.2.1, 2.2.2.2).

It is therefore the aim of this study to further elucidate the interaction effect of genetic, environmental and epigenetic factors on the HPA-axis stress reactivity.

2.1.2.3 The Detrimental Effects of Early Stressful Live Events

Which are the processes that facilitate the above-mentioned association between SLE and depressive symptomatology? Rodent, monkey, and human observations support hypothesis of an early programming of the endocrine stress response, through changes in corticolimbic structures involved in the regulation of the HPA-axis. Findings in rodent experiments, which manipulate the pre- and postnatal environment, consistently support an effect on the HPA-axis (Levine, 2005; Lupien et al., 2009; McEwen, 2007). In the womb, maternal circulating GC are able to infiltrate the fetal blood stream through the placenta (Lupien et al., 2009) and lead to brain alterations, such as reduced density of GR and MR in the hippocampus (reduced feedback inhibition) and increased CRH receptor density in the

amygdala (resulting in increased excitability) (de Kloet et al., 2005; Lupien et al., 2009). In consequence, these animals exhibit increased anxiety- and depression related behavior as adults (Lupien et al., 2009). Postnatal manipulation through maternal separation, handling, and other paradigms during sensitive phases (e.g. the first two weeks of life) (de Kloet et al., 2005) result in similar alterations, such as a decreased hippocampal GR density, upregulation of CRH binding sites in the amygdala, PFC, hypothalamus, and hippocampus (de Kloet et al., 2005; Lupien et al., 2009). In primates, postnatal stress exposure seems to parallel the rodent observations, leading to increased fear behavior and neurological changes in the hippocampus and PFC (Lupien et al., 2009).

Beside animals, human prenatal adversity such as maternal stress, depression, and anxiety during pregnancy is also associated with persistent alterations of the HPA-axis (Lupien et al., 2009). In addition, compared to animals, the human brain is uniquely characterized by its overall plasticity, especially during periods of growth such as the first year of life, in which it gains weight from 400g to 1000g (Glaser, 2000; Pittenger & Duman, 2007). Moreover, critical emotion regulation sites keep developing long after birth: relevant examples are the hippocampus (until age 2), the amygdala (until age 20), and the PFC (rapid growth during adolescence) (Lupien et al., 2009). Additional to these sensitive phases, the infant's stress system is not fully developed and prone to inadequate activation and an excess of GCs (Anda et al., 2006). High concentrations of GCs are discussed in the context of neural vulnerability or death, possibly leading to hippocampal atrophy (Lee, Ogle, & Sapolsky, 2002; Sapolsky, 2000b). These assumptions are heavily supported by retrospective ESLE studies that report diverse alterations of structures relevant to cognition, memory, and emotion, hence HPA-axis regulation (Agid et al., 2000; Beers & De Bellis, 2002; Bremner, 2003; Glaser, 2000; Lee et al., 2002; Margolin & Gordis, 2000). Accordingly ESLE have been observed in associated with increased HPA-axis (Gunnar & Quevedo, 2007; Heim & Nemeroff, 2001; Heim et al., 2001, 2004; Heim, 2000; Kaufman et al., 2000; Lupien et al., 2009), but also blunted HPA-axis reactivity (Burke et al., 2005; Lovallo et al., 2012; Lupien et al., 2009; Strüber et al., 2014).

In conclusion, accumulating and converging evidence heavily supports the possibility of neurological alterations relevant to emotion and HPA-axis regulation by ESLE. Furthermore, a considerable part of the depressed population has experienced ESLE and also exhibits alterations in the HPA-axis reactivity to stressful stimuli. However, (E)SLE just account for a small part of the overall variance in psychopathology (Dohrenwend, 2006) and the reaction to these events differs considerably (Hoven et al., 2005). Consequently, there have to be other factors involved in the etiology of MDD and the workings of the endocrine system.

One of these, known through heritability studies, is the genetic component of MDD (Sullivan, Neale, & Kendler, 2000). Therefore the next section will illustrate the current state of research and a brief overview of the history and consequent rationale in the genetic investigation of the depressive disorder.

2.1.3 Genetics of Stress and Depression

Besides the association with stress, MDD also shows a moderate to high heritability of 31-50% in epidemiological and twin studies (Kendler, Thornton, & Prescott, 2001; Levinson, 2006; Nestler et al., 2002; Sullivan et al., 2000) and familial aggregation (OR 2.84) (Sullivan et al., 2000). Based on these findings, MDD seems to have a considerable genetic component, though the exact subjacent genetic mechanisms remain unknown (Flint & Kendler, 2014b). In recent years, genome wide association studies (GWAS) have been applied to detect risk genes or gene constellations, but have yet to yield success (Flint & Kendler, 2014a). Thus, scientists have to follow hints from empirical observations from related fields. To understand the choice of candidate genes, it is important to know that research hitherto orientate itself at the neurotransmitter theories of depression, which are grounded in psychopharmacology (Krishnan & Nestler, 2010; Owens & Nemeroff, 1994). When 5-HT was first discovered (under the name enteramine) in 1946 (Owens & Nemeroff, 1994), it had yet to be recognized as a transmitter in the brain. The structural similarity to lysergic acid diethylamide (LSD) (Owens & Nemeroff, 1994), led to the hypothesis of serotonin's contribution to mental states[5]. This possibility was supported by the accidental finding that the 5-HT, dopamine (DA), and NE synaptic concentration were increased by the pharmacological agents iproniazid (a monoamine oxidase inhibitor, MAO-I) (Owens & Nemeroff, 1994) and imipramine (tricyclic

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[5] The role of 5-HT in depression and emotional regulation (Neumeister et al., 2004; Ruhé et al., 2007, Van der Does, 2001) is further supported by tryptophan (an essential amino acid and precursor of 5-HT) depletion studies that observed a drop in mood in healthy subjects with a family history of depression (Owens & Nemeroff, 1994) and an increased relapsed risk in former depressed patients (Owens & Nemeroff, 1994). Moreover, reduced CSF concentrations of hydroxyindoleacetic acid (5-HIAA, the metabolic product of 5-HT) have been observed in depressed patients (Oberlander, 2012), and reduced concentrations of 5-HT and 5-HIAA in postmortem brain tissue of depressed and suicidal subjects. Finally more or less all efficacious ADs achieve their impact by augmenting 5-HT concentration and doing this by inhibition of 5-HT reuptake or metabolization (Gaspar et al., 2003).

antidepressant), which lead to antidepressant effects (Klerman & Cole, 1965; Krishnan & Nestler, 2008). Subsequent pharmacological development resulted in a new generations of ADs, the today popular selective serotonin reuptake inhibitors (SSRI) and MAO-I (Krishnan & Nestler, 2008). These psychotropic substances are believed to exert their effects by far-ranging alterations of the 5-HT system (e.g. transcriptional and structural changes) (Krishnan & Nestler, 2008; Vaswani, Linda, & Ramesh, 2003). However, it should be kept in mind that 5-HT is involved in a multiplicity of phenomena (e.g. innervation of the digestive system, sleep, learning, memory, pain, sex, motor activity, biological rhythm, and neuroendocrine regulation) (Zifa & Fillion, 1992), psychiatric disorders (e.g. depression, anxiety-, panic- and eating disorder, as well as psychosis) (Naughton, Mulrooney, & Leonard, 2000), interacts with 14 different receptor types grouped in 7 families (Göthert, 2013), and therefore there "is no simple direct correlation of 5-HT [...] levels in the brain and mood" (Ruhé et al., 2007, p. 354).

Nonetheless, 5-HT is crucial to this thesis because of its influence on the HPA-axis (Dinan, 1996; Heisler et al., 2007) and its important role in early neuronal development and maturation[6] (Gaspar, Cases, & Maroteaux, 2003; Nguyen et al., 2001; Oberlander, 2012). To support this viewpoint, critical changes in stress regulating structures associated with a genetic variant believed to influence 5-HT levels are discussed further below (2.1.3.1).

Coming back to the search for candidate genes in MDD, the pursuit of genetic factors involved in the 5-HT system, led amongst others to the *SLC6A4* (Levinson, 2006). It is the single gene that encodes for the SERT. This protein, located in the presynaptic membrane, influences 5-HT availability via 5-HT reuptake and thus acts as a intrasynaptic 5-HT master controller (Nakamura et al., 2000; Oberlander, 2012). A functional length polymorphism in the promoter region of the *SLC6A4* is the single most researched genetic variation in the fields of psychiatry, psychology, and neuroscience (Caspi, Hariri, & Holmes, 2010) and is of special interest due to its association with depression, neuronal development, and HPA-axis reactivity (Caspi et al., 2010; López-León et al., 2008; Miller et al., 2012; Oberlander, 2012).

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[6] 5-HT is a phylogenetic old transmitter that is found throughout the brain (Oberlander, 2012) and is released by growing axons before synapse are established (Oberlander, 2010). 5-HT acts as a trophic factor on "cell division, differentiation, migration, myelination, synaptogenesis, and dendritic pruning" (Oberlander, 2012), consequently influencing the organization of the 5-HT and other systems (Oberlander, 2012). Importantly 5-HT is essential in HPA-axis functioning and development (Oberlander, 2012) and the described early neurological modifications (2.1.3.2) might in part be explained by altered 5-HT availability.

2.1.3.1 The Serotonin-Transporter Length Polymorphism

The *SLC6A4* is mapped at the chromosomal loci 17q11.1-q12 (Heils et al., 2002; Nakamura et al., 2000) and in its upstream regulatory promoter region, multiple functional polymorphisms have been detected (Wendland, Martin, Kruse, Lesch, & Murphy, 2006). One of these, first described by Lesch (1996) and Collier et al. (1996), is a 43bp insertion/deletion (INDE) mutation in the *SLC6A4* gene-linked polymorphic region (5-HTTLPR) that is functionally relevant (Lesch et al., 1996; Wendland et al., 2006). The initial classification distinguished a long variant (L allele) with 16 repetitions and a short variant (S allele) with 14 repetitions (Lesch et al., 1996). The L allele was found to be associated with a threefold higher *SLC6A4* mRNA transcription efficiency in vitro lymphoblast cells, compared to the shorter variant (Collier et al., 1996). Though these findings could be replicated in several studies (Bradley, Dodelzon, Sandhu, & Philibert, 2005; Greenberg et al., 1999; Stoltenberg et al., 2002), research in vivo leaves significant doubt on a direct effect of one or two copies of the S allele on SERT availability. Peripheral blood mRNA concentrations in human (Yu et al., 2010) and animal studies (Singh et al., 2012) could not confirm an effect of the 5-HTTLPRgenotype on *SLC6A4* mRNA transcription efficiency. And though some positron emission tomography- (PET) and single-photon emission computed tomography (SPECT) studies found increased ligand binding to SERT proteins (Heinz et al., 2000; Praschak-Rieder et al., 2007; Reimold et al., 2007), the majority did not (Murthy et al., 2010; Parsey et al., 2006; Shioe et al., 2003; van Dyck, 2004; Willeit et al., 2001). These inconsistencies might be in part explained by epigenetic influences (2.2.2, 2.2.2.1), methodological heterogeneity (instruments, biomarkers), and various later discovered variants of the 5-HTTLPR (Nakamura et al., 2000). One of these is the subdivision of the L allele into a L_G and L_A version, caused by a single nucleotide polymorphism (SNP) that substitutes an adenine- for guanine base (Hu et al., 2006; Parsey et al., 2006). The L_G version has shown to be transcriptionally similar to the S allele, resulting in an tri-allelic model (L_A, L_G and S) (Hu et al., 2006), which has so far only been integrated in some studies.
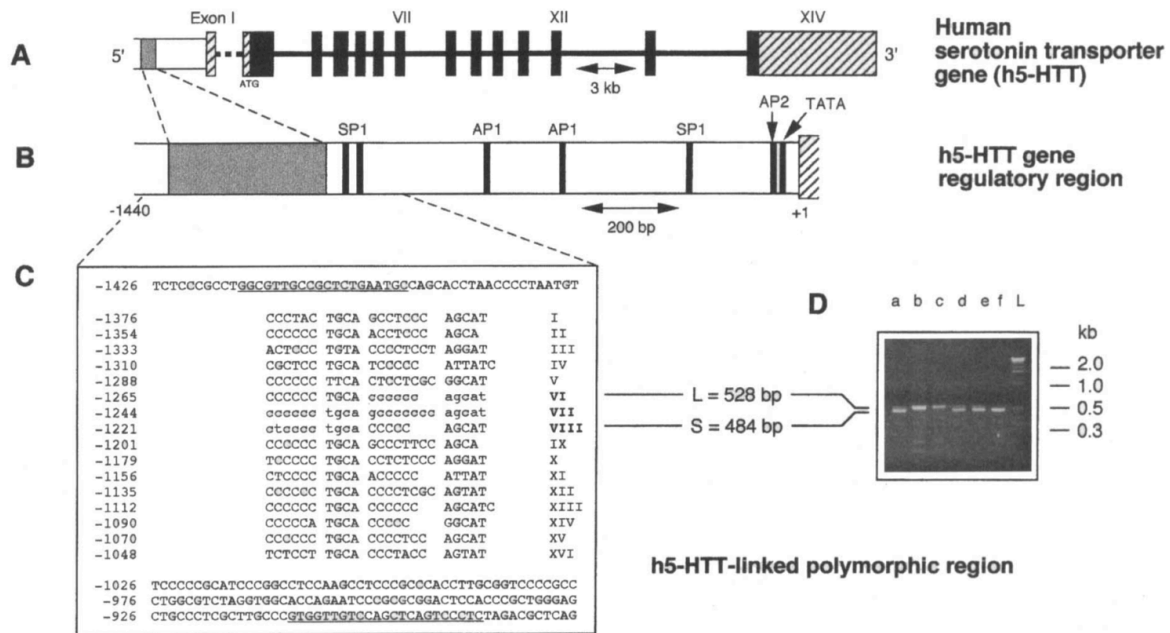
Figure 2 The *SLC6A4* gene and the 5-HTTLPR (Heils et al., 2002) **A**: Graphic representation of the *SLC6A4* and its flanking 5' regulatory region. Non-coding regions are depicted as solid boxes respectively coding regions as striped. **B**: Magnification of the 5'-flanking regulatory region of the *SLC6A4*. **C**: Nucleotide sequence flanking the 5-HTTLPR. Deletion elements are depicted in lower case letters. **D**: Polymerase chain reaction products (S- and L allele).

Despite conflicting observations (Lasky-Su, Faraone, Glatt, & Tsuang, 2005), the low transcribing alleles (S/L_G) have a small, but significant association with depression (Clarke et al., 2010; López-León et al., 2008) and the corresponding personality trait neuroticism (overview see: Canli & Lesch, 2007; Schinka, Busch, & Robichaux-Keene, 2004; Sen, Burmeister, & Ghosh, 2004). Though it should be mentioned that considerable controversies and inconsistencies still exist in the field (Canli & Lesch, 2007; Munafò, Clark, & Flint, 2005). An approach to solve the widespread problems of genetic research in complex psychiatric disorders is the use of intermediate internal phenotypes that are believed to have a simpler genetic architecture then mental phenomenon (Gottesman & Gould, 2003; Meyer-Lindenberg & Weinberger, 2006; Rasetti & Weinberger, 2011).

2.1.3.2 The Use of Endophenotypes in Genetic Research

The phenotypes we observe (e.g. MDD) are constituted by an ongoing dance of day-to-day interplay of environmental influences, pleiotropic gene effects, reciprocal gene on gene actions, gene by environment interactions, and epigenetic modifications (Gottesman & Gould, 2003; Hasler, Drevets, Manji, & Charney, 2004; Meyer-Lindenberg & Weinberger, 2006;

Rasetti & Weinberger, 2011). Alongside the difficulty of grasping this web of multi dynamic interactions, research has to rely on a nosological system (Diagnostic and Statistical Manual, DSM) that is based on clusters and characteristics of phenotypes that lack biological classification and are under constant revision (Gottesman & Gould, 2003; Hasler et al., 2004; Meyer-Lindenberg & Weinberger, 2006).

A suggested strategy to address these methodological problems and the small effect sizes of single gene variations in psychiatric disorders is the use of so-called endophenotypes. Endophenotypes represent intermediate, internal phenotype that are located downstream of the observable phenotype (e.g. depression) and upstream of a gene and it's transcriptional product (e.g. *SLC6A4*): from genes − mRNA − proteins − cells − to organs − circuits − the emergence of consciousness and mental disorders (Rasetti & Weinberger, 2011). The idea is that levels more 'proximate' to the DNA transcription products are determined by less genetic factors, resulting in a simpler genetic architecture compared to, for example intricate mental phenomena (such as emotions and disorders) (Canli et al., 2006). Gottesman and Gould (2003) adapt five criteria for the identification of endophenotypes in psychiatric disorders. *First*, the endophenotype has to show an association with illness. *Secondly*, it must be heritable. *Thirdly*, the endophenotype is independently of illness present. *Fourthly*, in families, endophenotypes and illness co-segregate. *Fifthly*, the endophenotype has a higher prevalence in non-sick family members than in the general population. The aspired benefit of this approach is an increase in statistical power (Gottesman & Gould, 2003; Hasler et al., 2004; Meyer-Lindenberg & Weinberger, 2006; Rasetti & Weinberger, 2011), the avoidance of a non biological based classification system (Meyer-Lindenberg & Weinberger, 2006) and the improvement of measurement precision by the use of endocrinological, biochemical, neuroanatomical, cognitive or neuropsychiatric indicators (Gottesman & Gould, 2003).

2.1.3.3 A Link Between Stress, the 5-HTTLPR, and Depression – HPA-axis

A central approach of this thesis is to use HPA-axis reactivity as a link between Depression, the 5-HTTLPR and early traumatization. This rationale is based on research linking alterations of the HPA-axis stress reactivity to genetic (5-HTTLPR) and environmental (ESLE) factors. Furthermore, the HPA-axis has been successfully used as an endophenotype of MDD (Flint & Munafò, 2007; Hasler et al., 2004) and is also interconnected with genetic and environmental factors. The HPA-axis fulfills the above mentioned criteria of heritability, co-

segregation, state independence, and familial aggregation (Gottesman & Gould, 2003; Hasler et al., 2004; Miller et al., 2012). Additionally, diverse neurological alterations in structures involved in the regulation of the cortisol stress response have been observed in depressive disorders (Heim & Nemeroff, 2001; Heim, Newport, et al., 2008) and as a result of ESLE.

First, an imbalance of MR/GR ratio has been suggested as a characteristic for stress related disorders and might result in reduced feedback inhibition of the HPA-axis (overview see: Hasler et al., 2004). *Secondly*, ADs are assumed to partly exert their effects by enhancing HPA-axis negative feedback inhibition through an increase in GR/MR expression and functioning (Pariante, Thomas, Lovestone, Makoff, & Kerwin, 2004). *Thirdly*, the DEX/CRH test has a sensitivity of 80% to distinguish between psychiatric patients (such as mania, schizophrenia, and MDD) and healthy subjects (Hasler et al., 2004). *Fourthly*, an abnormal cortisol response has been observed in high risk and MDD patients (overview see: Hasler et al., 2004). *Fifthly*, cortisol interacts with neurotransmitters, neuropeptides and brain circuits[7], which are associated with depressive symptomatology (Gold, Drevets, & Charney, 2002).

Coming back to the initial topic, the early assumption that the 5-HTTLPRgenotype exerts its influence over SERT availability could not be confirmed (2.1.3.1). It has therefore been hypothesized that the 5-HTTLPR could influence neural developmental, in which 5-HT plays a crucial role, especially for the corticolimbic system (Caspi et al., 2010; Homberg & Lesch, 2011). A convincing argument for this reasoning is the case of genetically engineered SERT 'knockout' mice, whose *SLC6A4* is rendered useless (Holmes, Murphy, & Crawley, 2003). These animals exhibit anxiety and depression like behaviors, impaired fear extinction, and an increased emotional and endocrine sensitivity to stress (Caspi et al., 2010; Gross & Hen, 2004; Holmes et al., 2003; Homberg & Lesch, 2011; Karabeg et al., 2013). Importantly, these effects can be partially mimicked, if the *SLC6A4* is pharmacologically blocked

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[7] These circuits include: a) reduced volume in the part of the PFC involved in the inhibition of the amygdala and HPA-axis activity – in turn leading to an increase in cortisol – which itself inhibits the control, attention regulation, and planning of the PFC (Gold et al., 2002); b) increased metabolism in the left amygdala, which inter alia inhibits the working of the PFC and activates the HPA-axis and the ANS. Additionally, corticosteroids enhance amygdala activity and increase CRH's effect on fear conditioning (Gold et al., 2002); c) reduced hippocampal volume, which seems to be positively correlated with the duration of depression – leading to decreased feedback inhibition (Gold et al., 2002). In summary, the common finding of HPA-axis alterations and dysregulation of diverse self-regulatory circuitries observed in MDD predestine the endocrine stress response as an ideal endophenotype to pursue in the investigation of depressive disorder.

temporarily during the first two weeks of life (Gross & Hen, 2004). This fact strongly supports a developmental, instead of a continuous influence of the 5-HTTLPR.

The first study to use the endophenotype approach, was by Hariri et al. (2002), who observed a hyperreactivity of the amygdala towards angry faces, in S allele carriers compared to individuals homozygous for the L allele. It was hypothesized that this could contribute to the susceptibility to affective disorders (Hariri et al., 2002). These findings were later confirmed in a larger sample (Hariri et al., 2005) and a recent meta analysis by Murphy et al. (2013). Subsequent studies focusing on the amygdala and associated brain circuits confirmed the role of the 5-HTTLPR in emotional processing (Bertolino et al., 2005; Canli et al., 2005; Furmark et al., 2004; Hariri & Holmes, 2006; Hariri et al., 2002, 2005; Heinz et al., 2005; Osinsky et al., 2008; Pezawas et al., 2005). Moreover, several studies using heterogeneous methods (review see: Canli & Lesch, 2007) reported convergent results in changes of structure, functioning, and interconnectedness of emotional processing networks in healthy (Canli et al., 2005; Furmark et al., 2004; Heinz et al., 2005; Pezawas et al., 2005), as well as clinical populations (Bertolino et al., 2005).

These networks are of crucial importance in regulating the HPA-axis response to stress (2.1.1). Convergent, recent meta-analysis confirm an effect of the short allele (especially in the S/S genotype) on increased HPA-axis reactivity (Gotlib, Joormann, Minor, & Hallmayer, 2008; Miller et al., 2012; Way & Taylor, 2010). However, the discovered effect sizes are small (Gotlib et al., 2008; Miller et al., 2012) and Miller et al. note that "even the largest study to date was underpowered to detect the small effect revealed by this meta-analysis" (Miller et al., 2012, p. 5). They concluded that effect sizes might increase, if environmental predictors would be additionally taken into account (Miller et al., 2012). Supporting this assumption is a recent PET study by Kalbitzer and colleagues (2010), who report a significant effect of the S allele on in vivo cerebral SERT binding, but only if season (environmental factor) was taken into account. This influence of context on genes is also the translation into the next topic, which is gene by environment interaction.

2.2 Gene by Environment Interaction

As discussed, genetic (2.1.3) as well as environmental (2.1.2) factors contribute to HPA-axis stress reactivity and mental disorders. But the individual reaction to, and further development after (E)SLE is heterogenic (Klengel & Binder, 2013). Furthermore, is the contribution of single gene variant in complex psychiatric disorders likely minor, since over 100 gene polymorphisms are estimated be involved (Canli & Lesch, 2007). Modern approaches therefore increasingly try to consider the complex interdependence of genomic predisposition and environmental pathogens. Though the idea of a gene by environment (G × E) interaction has its roots in the early 20th century (Kraft & Hunter, 2005), the first interaction studies investigating a single gene loci were conducted in the beginning of the 21st (Caspi et al., 2002, 2003; Munafò, Zammit, & Flint, 2014). In 2003, Caspi and colleagues (Caspi et al., 2003) assessed the effects of the 5-HTTLPRgenotype, stressful experiences, and their interaction on depressive symptoms in a well-controlled prospective longitudinal study (age 3 – age 26) consisting of n = 847 Caucasian participants. A major finding was that recent and ESLE (< age 10) more reliably predicted a later depressive episode if one or two copies of the S allele were present (Caspi et al., 2003). This highly influential (3000 < citations) and potentially paradigm shifting study (Caspi et al., 2010; Risch et al., 2009) set of a wave of subsequent research in neuroimaging, rodent, primate, and human studies in motion (Wankerl et al., 2010). The approach to investigate candidate genes in combination with environmental pathogens to increase explained variance in hitherto often marginal effect sizes of single genes has been quickly embraced (Risch et al., 2009). But even though an initial literature review (Uher & McGuffin, 2008) and its update (Uher & McGuffin, 2010) reported positive replications, the first two meta-analysis (Munafò, Durrant, Lewis, & Flint, 2009; Risch et al., 2009) spoke against the existence of a G × E interaction effect. On the contrary, a third analysis by Karg et al. (2011) concluded there exists strong evidence for a G × E interaction effect. This notion is further supported by a literature review in the same year by Nugent, Tyrka, Carpenter, & Price (2011). These contradictions, and the resulting discussion on whether a G × E interaction exists, is subject of an ongoing intense scientific debate (Wankerl et al., 2010). It is important to note that the procedures and informational value of the first two meta-analyses have been criticized for their pure statistical approach (Caspi et al., 2010; Wankerl et al., 2010), their quality, and heterogeneity of methods (measurement methods,

definition of SLE, samples, and classification of MDD) (Wankerl et al., 2010). In conclusion, most replication studies do not reach the high quality standards of the initial findings by Caspi et al. (Caspi et al., 2002, 2003; Wankerl et al., 2010). But following an inclusive approach, the converging evidence of the collated data of genetic, environmental, neurological, and animal studies support the notion of a joint contribution of genetic disposition and environmental stimuli (Wankerl et al., 2010).

2.2.1 Advancing Gene by Environment interaction research

G × E interactions are highly complex and constantly changing processes that are not limited to the momentary association of two variables (Homberg & van den Hove, 2012). To further advance G × E research and potentially solve the currently existing controversies, several approaches have been suggested, of which two converge with the hitherto presented data. Namely, the potential programming effects of ESLE during early developmental periods (Caspi et al., 2010) and the investigation of the HPA-axis reactivity to psychosocial stress as an endophenotype of MDD (Alexander et al., 2009).

2.2.1.1 Early Adverse Experience

After analyzing several studies and the Dunedin cohort data (which was also used in the study by Caspi et al., 2003), Brown and Harris (2008) concluded that the evidence for a G × E interaction is much stronger in the case of ESLE compared to SLE. This view is supported by the insight that recent (< 5 years) SLE have a smaller effect on depression onset if controlled for the existence of ESLE (Brown & Harris, 2008). This fact also underlines the uncertainty of a direct contribution of the 5-HTTLPR to SERT availability (2.2.1) and points in the direction of early neural adaptations similar to the alterations found in subjects who experienced ESLE (2.1.3.2). Furthermore, reports on animals, especially the impressive studies in *SLC6A4*-knockout mice, point towards sensitive phases in development that are open to adaption. Another point is the seeming paradox of the association of the S allele with anxiety, neuroticism, and depression. If the assumption of a direct effect of decreased SERT availability would be true, shouldn't this result in similar effects as SSRI that inhibit 5-HT reuptake? An influence of the 5-HTTLPRgenotype during neurodevelopment has therefore already been hypothesized in the original paper by Lesch et al. (1996) and is further supported

by the contribution of serotonin in neuronal genesis (2.2), the increased plasticity of the young brain, and the neurological endophenotypes (2.2.2.1) associated with the 5-HTTLPRgenotype (2.2.2.1).

A variety of recent G × E interaction studies successfully followed this approach: the initial findings of ESLE interacting with the 5-HTTLPRgenotype (Caspi et al., 2003) have inter alia been replicated to have an effect on depressive symptomatology (Aguilera et al., 2009; Fisher et al., 2013; Owens et al., 2012; Pauli-Pott, Friedel, Friedl, Hinney, & Hebebrand, 2009) and other studies report an increase in anxiety sensitivity in S allele carriers (Stein, Schork, & Gelernter, 2008), emotional responsiveness (Stein et al., 2008), fear behavior (Pauli-Pott et al., 2009), suicidal ideation (Cicchetti et al., 2010), panic disorder (Choe et al., 2013), and eating disorder (Akkermann et al., 2012). Though non-significant reports exist (Reinelt et al., 2013), inverted effects are found (Klauke et al., 2011), and other interactions (such as environment-environment sensitization by ESLE to later SLE) (Power et al., 2013) are detected, the success of recent studies support the implementation of the early developmental background in the prediction and understanding of psychopathology in the context of G × E interactions.

2.2.1.2 Endophenotypes in Gene by Environment Interaction Research

As has been argued (2.1.3.2) that endophenotypes pose the advantage to be more proximate to the DNA product and that they overall constituted by fewer variables then the surface phenotypes we directly observe. Genetics as well as environmental factors influence the physiological reaction to stress and it is therefore conceivable that the HPA-axis might integrate these (and other) variables in its functioning. Besides the aimed improve in statistical power through the reduction of confounding factors, the use of biological markers to indicate the reaction to stress comprises the advantage of an objective indicator compared to questionnaires for depression.

Already in 2004, Barr et al. (2004) could observe an G × E interaction effect between the rh5-HTTLPR (the 5-HTTLPR analogue in rhesus macaques) and early rearing conditions (maternal separation) on the HPA-axis stress response in monkeys. Interestingly, in females an effect of rh5-HTTLPRgenotype was only present if earlier adversity had been encountered (Barr et al., 2004). Likewise in humans, a joint contribution of the 5-HTTLPRgenotype and SLE could be reported for the first time in 2009 (Alexander et al.,

2009). Alexander et al., (2009) investigated the HPA-axis stress reactivity to the TSST in a sample of n = 100 healthy young men. Statistical analysis revealed no main effect of either SLE or the 5-HTTLPRgenotype (Alexander et al., 2009). But if the interaction between both variables was investigated, the analysis revealed increased cortisol secretion in individuals who experienced SLE and were additionally homozygotes for the S allele (Alexander et al., 2009). Another impressive finding of a G × E interaction on cortisol reactivity has been reported by Mueller et al. (2011). They investigated three different age cohorts (children, young adults, old adults) and differentiated the temporal occurrence of SLE (before the age of five, respectively 15 and overall SLE, Mueller et al., 2011). If SLE were not considered, the main effect analysis revealed a significant association between the individuals with the L/L-genotype and an increase in HPA-axis reactivity to stress (Mueller et al., 2011). Impressively, the authors were able report an interaction effect between SLE during the first five years of life and the 5-HTTLPRgenotype that reversed the influence of the S- and L allele on cortisol stress secretion (Mueller et al., 2011). Participants carrying one or two copies of the S allele showed an increased HPA-axis reactivity compared to L/L individuals (Mueller et al., 2011).

These results support an influence of SLE on HPA-axis reactivity and the importance of stressful experiences during the early stage of life. Particularly interesting is the evocation (Alexander et al., 2009), respectively reversal (Mueller et al., 2011) of the effect of the 5-HTTLPRgenotype by incorporation of SLE measures, which underlines the importance of recording both genetic and environmental information. A final argument in support of the use of endophenotypes in G × E interaction research is a recent study by Klucken et al. (2013). The authors investigated the interaction between 5-HTTLPRgenotype and SLE on the neural processes in structures associated with fear conditioning (amygdala, thalamus, insula, and the occipital cortex) (Klucken et al., 2013). Individuals' homozygotes for the S allele, with a history of SLE, experienced an increased reaction to fear stimuli in the area of the insula and occipital cortex, compared to all other groups (Klucken et al., 2013). These results further highlight the proposed network of neurological structures involved in emotional processing and fear conditioning, the 5-HTTLPRgenotype, SLE, and the HPA-axis as a potential integrator of these phenomena.

Though these findings need further replication, these initial results highlight the usefulness and logical stringency of using biological endophenotypes in G × E interaction research.

2.2.2 A Molecular Mechanism: Epigenetic regulation through DNA methylation

Despite the importance of G × E interaction research, the molecular mechanisms that mediate these associations are unknown. Such an understanding is however pivotal if these findings should become beneficial to diagnostic, therapy, and prevention (Mill & Petronis, 2007). Promising contenders to fill this gap in understanding are epigenetic processes (from Greek 'epi', meaning upon) (Heim & Binder, 2012; Kinnally et al., 2011; Lesch, 2011; Meaney, 2010; Tsankova, Renthal, Kumar, & Nestler, 2007; Weaver, 2007). Epigenetic processes are defined as structural or chemical alterations to the DNA that influence the gene product without changes to the nucleotide sequence (Foley et al., 2009; Meaney, 2010). They are dynamic, especially during early embryogenesis, but can also be modulated in fully differentiated mature cells and neurons (Foley et al., 2009; Klengel, Pape, Binder, & Mehta, 2014). Epigenetic marks can be maintained through mitosis and have so far been observed to be meiotically heritable in animals (Foley et al., 2009). Notably the epigenome is influenced by environmental agents, such as diet, smoking, alcohol, heavy metals, pesticide, and for psychological research most relevant social interaction (e.g. maternal behavior) (Bagot & Meaney, 2010; Champagne & Curley, 2005; Foley et al., 2009; Rutter, Moffitt, & Caspi, 2006; Szyf, 2013; van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010; Zhang & Meaney, 2010). Further, DNA methylation seems to be also influenced by the underlying DNA sequence (Hellman & Chess, 2010). It has been shown that about 10% of the common allelic variants are associated with a change in DNA methylation (Hellman & Chess, 2010) and that methylation might be influenced by subjacent gene motifs and splice variants (Vijayendran, Beach, Plume, Brody, & Philibert, 2012).

To date, the best researched of these processes is DNA methylation (Foley et al., 2009). Other known epigenetic processes include chromatin remodeling via histone modification and non coding micro RNA (Foley et al., 2009). DNA methylation is the addition of a methyl group to a cytosine base by one-carbon metabolism from a methyl donor, which is catalyzed by the enzyme DNA methyltransferase (Allis, Jenuwein, & Reinberg, 2007; Foley et al., 2009; Jones & Baylin, 2002; Kaffman & Meaney, 2007). DNA methylation preferentially occurs at so-called CpG sites (a cytosine nucleotide neighbored by a guanine nucleotide and connected via a phosphate group in the backbone of the DNA) (Allis et al., 2007; Bagot & Meaney, 2010; Foley et al., 2009). Regions containing a high density of CpG dinucleotides are called CpG

islands (Foley et al., 2009). Predictions estimate that around 29.000 of these islands exist in the human genome (Bird, 2002). They are associated with approximately 60% of human genes and are often times located in their 5' ends regulatory regions (Bird, 2002).
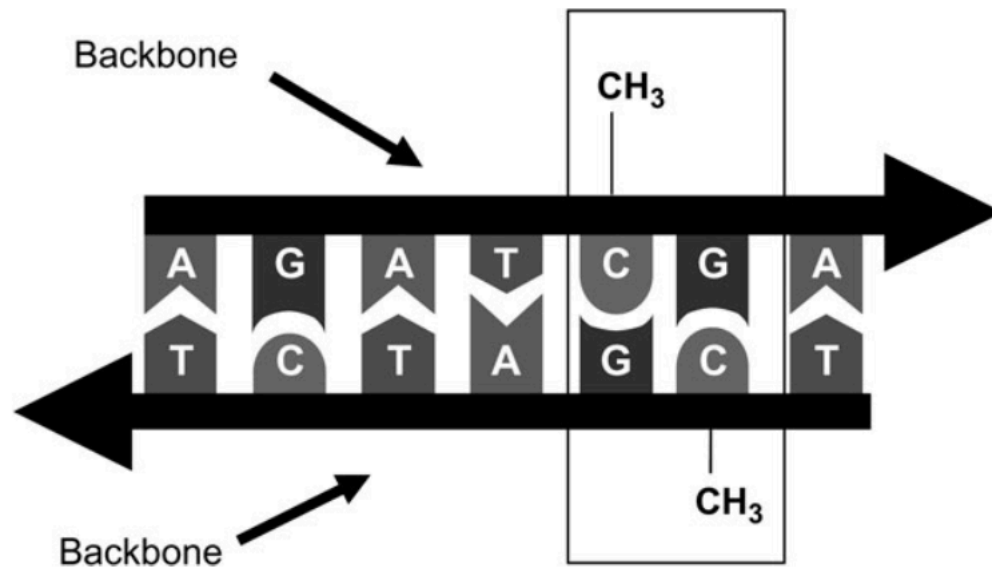
Figure 3 DNA methylation Simplified representation of the addition of methyl groups (CH_3) at the backbone of the DNA at CpG dinucleotides (Foley et al., 2009)

Until recently, DNA methylation was thought to only occur during early fetal development in the context of X-chromosome inactivation in females and parental gene imprinting (Allis et al., 2007; Foley et al., 2009). Instead, it has now become clear that DNA methylation can occur in mature cells and neurons, is mitotically and potentially meiotically heritable, is overall dynamic, and influenced by environmental signals (Allis et al., 2007; Bjornsson et al., 2008; Foley et al., 2009; Kappeler & Meaney, 2010; Meaney & Szyf, 2005; Weaver, 2007). Increased levels of DNA methylation in regulatory regions of genes, such as promoters and enhancers, are associated with gene silencing (Allis et al., 2007; Foley et al., 2009; Tsankova et al., 2007). Methylated regions draw in methylation-DNA binding proteins, which further attract clusters of proteins known as repressor complexes that are the facilitators of gene silencing (Meaney, 2010). The functional relevant degree of methylation necessary for silencing to occur has not yet been pinpointed (Foley et al., 2009). Differences between affected and unaffected individuals can vary dramatically from e.g. 100% in cancer patients, to 10% in other so far investigated complex diseases (Foley et al., 2009). "However, for some genes, evidence exists that a small change in the level of DNA methylation, especially in the lower range, can dramatically alter gene expression" (Foley et al., 2009, p. 319). Gene silencing can either occur by either 'broadband' methylation of big regions of DNA, thus

preventing transcription through density of DNA methylation, or the likely more common process of specific methylation of CpG sites in dynamic regions, such as the brain (Zhang & Meaney, 2010).

The gold standard to quantify DNA methylation in the region of interest is sodium bisulfite treatment (Foley et al., 2009). This process converts unmethylated cytosines to uracil (Herman & Graff, 1996). These changes can then be assessed by either DNA sequencing, polymerase chain reaction amplification, or mass spectroscopy (Foley et al., 2009).

2.2.2.1 DNA Methylation in Gene by Environment Interaction Research

The so far unrecognized layer of information encoded in DNA methylation patterns poses exciting possibilities to improve our basic understanding of G × E interactions (Meaney, 2010). This additional dimension could contribute in solving the hitherto presented inconsistencies (2.2) and increase the predictive power of our current models. A molecular mechanism facilitating gene-environment 'communication' could improve our understanding on how ESLE influence neuronal development (2.1.2.3) (Bagot & Meaney, 2010; Heim & Binder, 2012; Mehta et al., 2013; Zhang & Meaney, 2010), how individual differences in stress reactivity emerge in an environmental context (2.1.2.2) (Harper, 2005; Tsankova et al., 2007; Weaver et al., 2004), and it might improve the small, but consistently observed effect sizes of the 5-HTTLPRgenotype on depression and stress reactivity (Clarke et al., 2010; Miller et al., 2012).

Therefore, DNA methylation is an ideal candidate to improve our understanding of G × E interaction as has been suggested by leading scientist of the field (Homberg & Lesch, 2011; Meaney, 2010; Yehuda et al., 2010). In summary, DNA methylation is a molecular and potentially functionally relevant modification of the DNA molecule. It is influenced by environmental signals, as well as the DNA structure, it is dynamic and therefore flexible to adept, and might be heritable.

2.2.2.2 DNA Methylation – Current State of Research

This section will give a brief overview of epigenetic research in the presented fields, focusing on studies investigating methylation in the *SLC6A4* promoter-associated region. Overall epigenetic research into psychiatric disorders is still in an embryonic state. Hence, analyzing methods (e.g. pyrosequencing, microarray, enrichment-based methods) and software (e.g. BRAT, Beadscan), as well as the investigated tissue material (e.g. peripheral blood, central nervous system [CNS] materials, lymphoblast cells, buccal cells, saliva) show a high level of variety (Bock, 2012; Klengel et al., 2014).

Depression has been found to be associated with the methylation state in the promoter-associated region of the *SLC6A4* (Kang et al., 2013; Kim et al., 2013; Philibert et al., 2008; Zhao, Goldberg, Bremner, & Vaccarino, 2013) and this relationship was further moderated by 5-HTTLPRgenotype in a study by Olsson et al. (2010).

Concerning effects of the 5-HTTLPRgenotype on DNA methylation, a potential contribution to DNA methylation is suggested by a study in rhesus macaques that found genotype dependent methylation differences (higher in S allele carriers) (Kinnally et al., 2010). These findings have not been replicated in humans, though Philibert et al. (2007) found a trend of higher *SLC6A4* methylation in S allele carriers.

A central characteristic of the DNA methylation state is its potential modification by environmental factors. This is convincingly affirmed in humans by a prospective longitudinal study investigating 28.000 CpG sites of 14.000 genes, which reported persistent overall alterations of DNA methylation dependent on parental stress measures during the first five years of life (Essex et al., 2013). Moreover, in a longitudinal study with n = 111 individuals, 8-10% exhibited a 20% change in their global methylation levels over a time span of $11 - 16$ years (Bjornsson et al., 2008). Furthermore, monozygotic twins with identical methylation profiles at birth, show a considerable 'epigenetic drift' (differences in DNA methylation and other epigenetic markers) later in life (Fraga et al., 2005; Martin, 2005; Wong et al., 2014).

As has been argued (2.1.2.2, 2.1.3.3, 2.2.1.2), HPA-axis stress reactivity is an endophenotype linking environmental, genetic, and potentially epigenetic factors to MDD. The possibility of a programming effect of the early environment (e.g. maternal care) on stress reactivity of the HPA-axis via DNA methylation (e.g. *GR*) has been impressively demonstrated by a highly influential series of rodent manipulation studies by Weaver and colleagues

(Weaver, 2007; Weaver et al., 2004, 2007; Weaver, Szyf, & Meaney, 2002). Furthermore, could a first study by Edelman et al. (2012) partially replicate these findings in a human sample. Edelman et al. (2012) investigated 39 CpG sites in the human *GR* promoter region. Interestingly, methylation levels at a single site (CpG 12) accounted for 28% of total female cortisol output. This result supports the influence of specific epigenetic modifications and their influence on the endocrine stress reactivity in humans.

Regarding methylation of the *SLC6A4* promoter-associated region, hitherto studies focused on a variety of psychiatric disorders and stressors (overview see: Klengel et al., 2014), such as depression (Alasaari et al., 2012; Kang et al., 2013; Zhao et al., 2013), post stroke depression (PSD) (Kim et al., 2013), burnout (Alasaari et al., 2012), sexual abuse (Beach, Brody, Todorov, Gunter, & Philibert, 2011; Vijayendran et al., 2012), childhood adversaries (Beach, Brody, Todorov, Gunter, & Philibert, 2010; Kang et al., 2013), antisocial personality disorder (ASPD) (Beach et al., 2011), and PTSD (Koenen et al., 2011). The most consistent pattern is an influence of ESLE (such as sexual and physical abuse) on increased levels of methylation in that region (Beach et al., 2011, 2010; Kang et al., 2013; Ouellet-Morin et al., 2013; Vijayendran et al., 2012).

An interesting theme in these studies is the interaction between *SLC6A4* promoter-associated region methylation and the 5-HTTLPRgenotype. As mentioned earlier, the short variants (S/L_G) of the 5-HTTLPR have been associated with reduced 5-HTT mRNA transcription, depression, neuroticism (2.1.2.1), and structural, as well as functional alterations (2.1.2.1). The emerged inconsistencies and small effect sizes (2.1.2.1) might be in part explainable, if epigenetic modifications are additionally taken into account. Interestingly, Van IJzendoorn et al. (2010) reported an increased risk for unresolved responses to loss or trauma in carriers of the usually 'protective' L allele homozygotes, if DNA methylation in the SERT promoter-associated CpG islands was relatively high. Conflictingly, in the case of relatively low DNA methylation levels, the S allele was associated with higher levels of unresolved loss or trauma (van IJzendoorn et al., 2010). In contradiction to these results, increased methylation levels were reported to increase the susceptibility to PSD (Kim et al., 2013) and the association of sexual abuse to ASPD in S/S allele carriers (Beach et al., 2011). Not concerning the 5-HTTLPR, but moderating susceptibility, Koenen et al. (2011) found that low *SLC6A4* promoter region methylation led to an increased risk for PTSD in interaction with traumatic events. These preliminary results illustrate the potential role of DNA methylation in fine-tuning the organism's reaction to environmental stimuli and that this

modification might interact with hitherto investigated effects of single genes and polymorphisms.

Regarding the presented endocrinological changes in reaction to early adversity (2.1.3.2), a first study by Ouellet-Morin et al. (2013) investigated changes in DNA methylation in the *SLC6A4* promoter-associated region and HPA-axis stress reactivity. In this longitudinal study design, non-bullied and bullied monozygotic twins were compared based on their methylation status and cortisol reaction to the TSST (Ouellet-Morin et al., 2013). The authors reported higher methylation levels in the bullied twin compared to his non-bullied sibling, which was further associated with a decreased cortisol response to the TSST (Ouellet-Morin et al., 2013). This result strongly supports the hypothesis of an epigenetically moderated association between early adverse experience and endocrinological stress reactivity.

Despite the exciting conclusions that one can deduct from the presented studies, these results await further replication and differ in not only in methodology, but also the investigated regions. There is still no consensus which region and which CpG sites to investigate. This is illustrated by the fact that analyzed procedures and reported associations range from single CpG sites to averaged results over the whole island of investigation.

2.3 Conclusion

Stress is a major risk factor in the etiology of depressive disorders and appears to be especially detrimental in developmental phases. Early stressful and traumatic experiences have been found to render the organism vulnerable to adverse experiences later in life. These findings might be in part attributable to a persistent alteration of the HPA-axis response to threat. Psychiatric genetics also identified gene variants that are associated with risk for MDD, such as the 5-HTTLPRgenotype. This INDE polymorphism in the promoter-associated region of the *SLC6A4* has amongst others been researched in the context of depression and also shows effects on the HPA-axis. Despite considerable improvements in methodology, both approaches have fallen short to explain the development of depression.

The modern paradigm of G × E interaction embraces the constant interplay of both, genetic and environmental factors and is an important progress in solving the discussed inconsistencies. Though, at this point in time, it has not yet produced clear-cut results. One approach to further advance G × E studies is the use of endophenotypes. In the case of the presented variables, the HPA-axis is an ideal object of investigation, since early trauma and the 5-HTTLPRgenotype influence it. First studies using the HPA-axis in G × E studies could proof the coherence of this rationale, but still reported small effect sizes. This might be in part due a lack of understanding of the molecular processes that transmit these effects. Now, epigenetic modifications, such as DNA methylation, are a potential mechanism facilitating the communication between environment and genome. They have been shown to be dynamic, but also stable alterations to the DNA sequence that influence the expression of genes, which in turn are able to effect downstream physiological systems. Importantly, they are sensitive to influences from the environment.

Accordingly, leading scientists in this field propose epigenetic processes as a promising subject of enquiry in the search of molecular facilitator of G × E interactions effects. It is therefore the goal of this study to investigate the effect of ESLE and the 5-HTTLPRgenotype on the methylation status of a 799 bp long CpG island in the promoter associated region of *SLC6A4*. In a second step, the influence of this methylation profile and its interaction with the 5-HTTLPRgenotype on HPA-axis reactivity (to psychosocial threat) will be examined.

3. Objectives and Hypotheses

It is the goal of the present study to investigate the effect of ESLE, the 5-HTTLPRgenotype, and the interaction of these variables on the methylation status in a 799 bp long CpG island in the promoter associated region of *SLC6A4* that has previously been defined by Philibert et al. (2008) (Fig 5). In a second step, the influence of this methylation profile and a potential interaction effect with the 5-HTTLPRgenotyp on HPA-axis reactivity to psychosocial threat will be examined.

```
4681  agctgagctcacatcccagccggtcagtcagataaacg¹catgggtatcg²agtactgctag
4741  gtcccaggaagaaagagagagcagctttcg³ggatggggacg⁴atggggaggtgtccg⁵aggt
4801  caagagaaagcg⁶gcacg⁷agcagacccctgtgtgccg⁸tcctgtgggcg⁹cg¹⁰gggcg¹¹gcaggg
4861  gaggcg¹²cacacctgctcctttgtgcagcctcccccctcccg¹³caaagtaaagagcaggaa
4921  agtcaggattcctcg¹⁴ctcg¹⁵gccctgccctgccg¹⁶gctgctccg¹⁷cg¹⁸ctccg¹⁹ctcctccctgc
4981  g²⁰agcg²¹tgtgtgtgtgtcg²²ggggtccctcccctcctggctctggggtcg²³ggcg²⁴cg²⁵cacccc
5041  g²⁶cccg²⁷tagcg²⁸cg²⁹gcccctccctggcg³⁰agcg³¹caaccccatccagcg³²ggagcg³³cg³⁴gagccg³⁵
5101  cg³⁶gccg³⁷cg³⁸gggaagcattaagtttattcg³⁹cctcaaagtgacg⁴⁰caaaaattcttcaagagc
5161  tctttggcg⁴¹gcg⁴²gctatctagagatcagaccatgtgagggcccg⁴³cg⁴⁴ggtacaaatacg⁴⁵gc
5221  cg⁴⁶cg⁴⁷ccg⁴⁸gcg⁴⁹cccctccg⁵⁰cacagccagcg⁵¹ccg⁵²ccg⁵³ggtgcctcg⁵⁴agggcg⁵⁵cg⁵⁶aggccagc
5281  ccg⁵⁷cctgcccagcccg⁵⁸ggaccagcctccccg⁵⁹cg⁶⁰cagcctggcaggtgggtccg⁶¹cttttcc
5341  tctccg⁶²cctcg⁶³aacccacg⁶⁴tttctttccagaccttcttccccg⁶⁵cctcg⁶⁶gggaggggggata
5401  gaaccg⁶⁷ctgcg⁶⁸ccccaccg⁶⁹ccctgcg⁷⁰aggaggcg⁷¹aggaggtgcatgcg⁷²ccccagcg⁷³gtgg
5461  gcg⁷⁴ccg⁷⁵gatcctgcccctgcg⁷⁶ccctccacg⁷⁷ctcagcaagagccagagctgaagctgaccg⁷⁸
5521  gccagagtgggagacg⁷⁹aggaacg⁸⁰tggagtgctcg⁸¹aagtgggcg⁸²ggcg⁸³taggggggctcctt
```

Figure 4 *SLC6A4* CpG Sequence The DNA sequence of the *SLC6A4* (GenBank accession number: NG_011747) promoter-associated CpG island as defined by (Philibert et al., 2008). The 83 CpG dinucleotides detected by bisulfite pyrosequencing (4.6) are highlighted in red (Wankerl et al., 2014)

Increased understanding of these areas could help to advance our understanding of the molecular mechanisms that communicate the effects of stress during early development on the organism (2.1.2.3). Moreover, increasing knowledge about epigenetic mechanisms could help to resolve observed inconsistencies and small effect sizes in research concerning the 5-HTTLPRgenotype (2.1.3.1), G × E interactions (2.2), and the programming process of the workings of the HPA-axis (2.1.3.3). The so far described associations and planned investigations are illustrated in the schematic overview below (Fig 5).
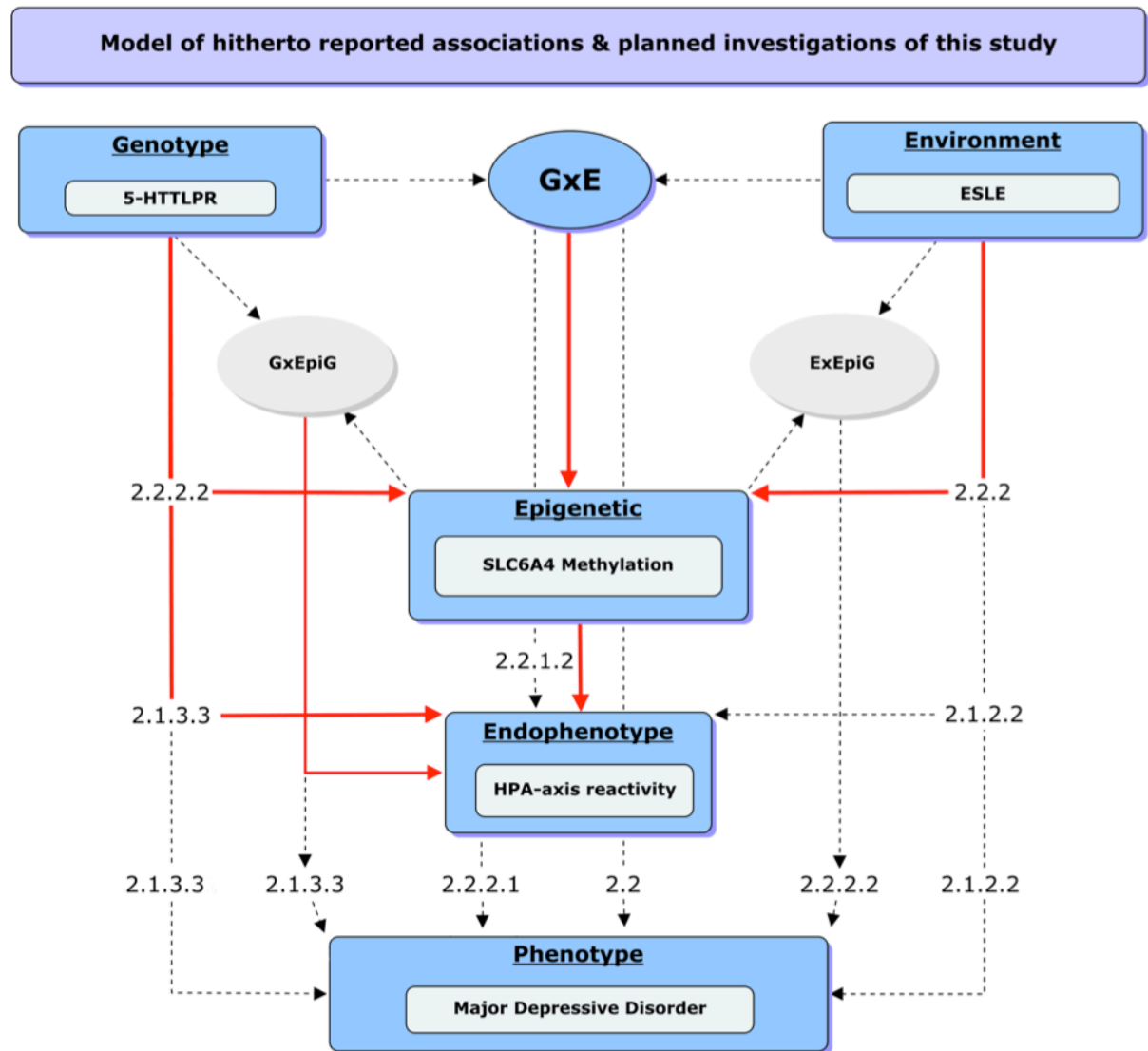
Figure 5 Schematic overview of the associations presented thus far: hypothesized epigenetic interactions (grey oval) and the planned investigations (red lines) in this study. Abbreviations: G × E, gene by environment interaction; ESLE, early stressful life events; G, gene; E, environment; EpiG, epigenetic; HPA-axis, hypothalamic-pituitary-adrenal axis.

3.1 Hypothesis 1A: Main Effect of ESLE on *SLC6A4* methylation

Decades of research have confirmed the long held notion that early adversity is associated with an increased risk to develop psychopathology later in life (2.1.2.2), yet the mechanisms communicating this vulnerability remain unclear. Epigenetic modifications, such as DNA methylation, are a potential molecular substrate to moderate these effects (2.2.2, 2.2.2.1, 2.2.2.2). This is primarily due to their dynamic response to signals from the environment. The features of MDD: to be a persistent and recurrent disease, its delayed response to AD treatment, and the neurological changes that characterize this disorder, all point towards a fundamental modification of the workings of the organism. Altered 5-HT availability, moderated by methylation in the *SLC6A4* promoter-associated region, might contribute to these changes during early developmental phases (2.1.3.2, 2.2, 2.2.2.1) and throughout life.

Initial studies in humans investigating the promoter-associated region of the *SLC6A4* could already successfully report an association between childhood adversity and methylation profile in its promoter region (2.2.2.2). Though the initial studies point towards an increase in CpG island methylation through early adversity, it would be premature at this moment to formulate a directed hypothesis.

$H_0: \mu ESLE_{\uparrow} = \mu ESLE_{\downarrow}$

$H1: \mu ESLE_{\uparrow} \neq \mu ESLE_{\downarrow}$

$\mu ESLE_{\uparrow}$:   mean of *SLC6A4* methylation in the high traumata group.

$\mu ESLE_{\downarrow}$:   mean of *SLC6A4* methylation in the low traumata group.

3.2 Hypothesis 1B: Main Effect of 5-HTTLPRgenotype on *SLC6A4* methylation

There exists evidence that DNA methylation can be influenced by the subjacent DNA sequence (Hellman & Chess, 2010). Moreover, there exists some evidence on the influence of the 5-HTTLPRgenotype (respectively the rh5-HTTLPR) on *SLC6A4* methylation (Kinnally et al., 2010; Philibert et al., 2007). Furthermore, it is a requirement to control for a direct

effect of genotype on *SLC6A4* methylation for the subsequent interaction effect analysis between the 5-HTTLPRgenotype and ESLE on *SLC6A4* mean methylation levels. Consequently, an effect of the 5-HTTLPRgenotype on *SLC6A4* methylation is investigated.

H_0: $\mu5\text{-HTTLPR}_\downarrow = \mu5\text{-HTTLPR}_\uparrow$

H_1: $\mu5\text{-HTTLPR}_\downarrow \neq \mu5\text{-HTTLPR}_\uparrow$

$\mu5\text{-HTTLPR}_\uparrow$:   mean of *SLC6A4* methylation with high expressing alleles (L/L_A).

$\mu5\text{-HTTLPR}_\downarrow$:   mean of *SLC6A4* methylation with low expressing alleles (S/L_G).

3.3 Hypothesis 1C: Interaction Effect of ESLE with 5-HTTLPRgenotype on *SLC6A4* methylation

A central objective of this study is to investigate a potential molecular mechanism that facilitates G × E interactions effects in the form of DNA methylation (2.2). The main point of reference of this thesis is the study by Caspi et al. (2003), which reported an increased susceptibility to depression in carriers of the S allele in the context of early adversity. Despite the relevance of these findings, the constituting molecular processes remain unclear. It is the suggestion of this thesis that DNA methylation might contribute to facilitate such effects. A joint effect of early traumatization with the 5-HTTLPRgenotype on *SLC6A4* methylation is therefore investigated.

H_0: $\mu5\text{-HTTLPR}_\uparrow\text{ESLE}_\uparrow = \mu5\text{-HTTLPR}_\uparrow\text{ESLE} = \mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\uparrow = \mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\downarrow$

H_1: $\mu5\text{-HTTLPR}_\uparrow\text{ESLE}_\uparrow \neq \mu5\text{-HTTLPR}_\uparrow\text{ESLE}_\downarrow \neq \mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\uparrow \neq \mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\downarrow$

$\mu5\text{-HTTLPR}_\uparrow\text{ESLE}_\uparrow$:   mean of *SLC6A4* methylation with high expressing alleles (L/L_A) and high traumata.

$\mu5\text{-HTTLPR}_\uparrow\text{ESLE}_\downarrow$:   mean of *SLC6A4* methylation with high expressing alleles (L/L_A) and low traumata.

$\mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\uparrow$:   mean of *SLC6A4* methylation with low expressing alleles (S/L_G) and high traumata.

$\mu5\text{-HTTLPR}_\downarrow\text{ESLE}_\downarrow$:   mean of *SLC6A4* methylation with low expressing alleles (S/L_G) and low traumata.

3.4 Hypothesis 2A: Main Effect of the 5-HTTLPRgenotype on HPA-axis reactivity

A small yet significant effect of the 5-HTTLPRgenotype on cortisol reactivity has been established in previous research (2.1.3). For this reason and to control for the assumption of independence between genotype and stress reactivity in the subsequent interaction analysis 2C, the main effect of the 5-HTTLPRgenotype on cortisol reactivity to the TSST is investigated.

H_0: μ5-HTTLPR$_\downarrow$ = μ5-HTTLPR$_\uparrow$

H_1: μ5-HTTLPR$_\downarrow$ ≠ μ5-HTTLPR$_\uparrow$

μ5-HTTLPR$_\downarrow$ mean of cortisol reaction in individuals with low expressing alleles (S/L$_G$).

μ5-HTTLPR$_\uparrow$ mean of cortisol reaction in individuals with high expressing alleles (L/L$_A$).

3.5 Hypothesis 2B: Main Effect of *SLC6A4* methylation on HPA-axis reactivity

A considerable body of literature in human and animal studies has established a programming effect of early environmental stress on the development of the HPA-axis reactivity to strain (2.1.2.3). An association between early traumatization and susceptibility to mental disorders, such as MDD, underlines the relevance of this effect (2.1.2.2). Functional epigenetic modifications constitute a potential molecular mechanism conveying the programming of the HPA-axis by early experiences. Consequently, an association between *SLC6A4* methylation and HPA-axis cortisol reactivity to the TSST is investigated.

H_0: μDNA methylation$_\uparrow$ = μDNA methylation$_\downarrow$

H_1: μDNA methylation$_\uparrow$ ≠ μDNA methylation$_\downarrow$

µDNA methylation↑	mean of cortisol reaction to the TSST in individuals with high *SLC6A4* methylation
µDNA methylation↓	mean of cortisol reaction to the TSST in individuals with low *SLC6A4* methylation

3.6 Hypothesis 2C: Interaction effect of 5-HTTLPRgenotype with *SLC6A4* methylation on HPA-axis reactivity

Since the HPA-axis is dysregulated in various mental disorders (2.1.1) and the 5-HTTLPRgenotype has been consistently found to influence its workings (2.1.3.2), it is hypothesized that DNA methylation might influence the HPA-axis stress reactivity in interaction with the 5-HTTLPRgenotype. Support for this reasoning stems from recent studies reporting *SLC6A4* methylation to moderate the relationship between 5-HTTLPRgenotype and psychopathology (2.2.2.2). The interaction of genetic with epigenetic factors on cortisol secretion to psychosocial stress is therefore investigated in a final step.

H0: µ5-HTTLPR↑5-HTTDNA methylation↑ = µ5-HTTLPR↓5-HTTDNA methylation↑ = µ5-HTTLPR↑5-HTTDNA methylation↓ = µ5-HTTLPR↓5-HTTDNA methylation↓

H1: µ5-HTTLPR↑5-HTTDNA methylation↑ ≠ µ5-HTTLPR↓5-HTTDNA methylation↑ ≠ µ5-HTTLPR↑5-HTTDNA methylation↓ ≠ µ5-HTTLPR↓5-HTTDNA methylation↓

µ5-HTTLPR↑5-HTTDNA methylation↑:	mean of cortisol reaction to the TSST in individuals with high expressing alleles (L/L$_A$) and high *SLC6A4* methylation
µ5-HTTLPR↓5-HTTDNA methylation↑:	mean of cortisol reaction to the TSST in individuals with low expressing alleles (S/L$_G$) and high *SLC6A4* methylation
µ5-HTTLPR↑5-HTTDNA methylation↓:	mean of cortisol reaction to the TSST in individuals with high expressing alleles (L/L$_A$) and low *SLC6A4* methylation
µ5-HTTLPR↓5-HTTDNA methylation↓:	mean of cortisol reaction to the TSST in individuals with low expressing alleles (S/L$_G$) and low *SLC6A4* methylation

4. Methods

4.1 Sample Description

An aim of the sample composition was to reduce confounding factors to the maximum extent. To achieve this, individuals had to go through several stages of selection. The final sample was composed of n=186 healthy Caucasian (96 female), German native speakers, between the age of 18 to 30. Subjects (balanced by gender, smoking status, contraceptive use, and employment status) were recruited via newspaper announces and flyers. A first screening phase was carried out via interviews (conducted by telephone- and in person). Exclusion criteria comprised the intake of HPA-axis influencing medications (e.g. asthma sprays, psychoactive drugs), a past or present diagnosis of a psychiatric disorder, a chronic physical health conditions (e.g. cardiovascular disease, cancer, diabetes), drug consumption, pregnancy, prior participation in a similar study, a BMI outside the range of 17-30, and the inability to forgo smoking for the duration of the test procedure (Appendix). Moreover, candidates underwent the Diagnostic Interview for Psychiatric Disorders—short version (Mini-DIPS). The Mini-DIPS is a structured interview that assesses point and lifetime prevalence of axis I disorders according to DSM-IV criteria. Subjects received information in advance on data privacy protection, the procedure of blood sampling, the expected expense allowance at the end of the study (50 €) and the declaration of consent. The study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Technische Universität Dresden.

4.2 Study Protocol

The study took place in four phases: Recruitment (via flyer and newspapers), initial screening (telephone interview), pre-appointment (further screening, administration of trauma checklist, and blood sampling) and main-appointment (TSST and cortisol measurement). After the screening phases and the signing of the informed consent, subjects received the Childhood Trauma Questionnaire (CTQ, 4.4.1), which is a checklist on early life traumatic experiences. Subsequently, a medical worker took blood samples for DNA genotyping and

DNA methylation profiling using Ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt) for storage and later DNA extraction. The EDTA tubes were stored for future analyses at -20C° for a maximum of 6 month. At the end of the pre-appointment, a date for the main-appointment was set. For female participants, the menstrual circle was determined and an appointment in the luteal phase (2.2.1) was arranged. The advantage of this two-staged approach was the avoidance of transfer effects due to HPA-axis response to the interview, questionnaire or blood sampling procedure.

On the day of the main appointment, subjects were welcomed and received general information about the upcoming study process and 'strain' test, as well as instructions on how to use the salivettes. Test subjects were then seated in a different room where they received a glass of grape juice to elevate blood glucose levels to facilitate a normal stress reaction (2.1.1).
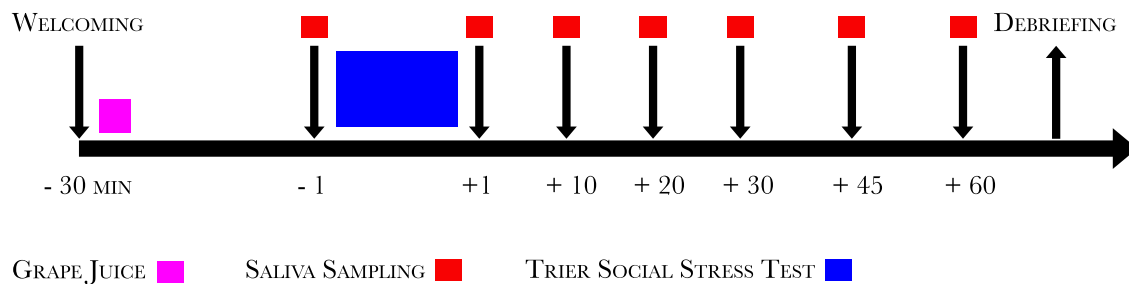
Figure 6 The TSST procedure

After half an hour and directly prior to the TSST, the first saliva sample (-1 min) was taken. Participants were then guided to the test room and asked to imagine their dream job (this was later used during the fictive job interview). Right after completing the TSST (see 4.3), subjects were guided back to their seat, where the second saliva sample was taken immediately (+1 min). Thereafter, further samples were taken at +10, +20, +30, +45, and +60 minutes after the test procedure (Fig 5). Upon completion, the test persons were debriefed, signed a fake letter of confidentiality, and received their compensation.

4.3 The Trier Social Stress Test

The Trier Social Stress Test is a standardized motivated performance task composed of a free speech and arithmetical task to induce psychosocial stress under laboratory conditions (Kirschbaum et al., 1993). Without previous instruction, the test person enters a room where he/she is being confronted with a seemingly professional evaluation committee. During a short introduction (see Appendix) by the test administrator, the two persons in the

room are pictured as professional behavioral observation psychologist and it is suggested that there will be video and voice recordings of the whole procedure. The subject is then seated at a table with a white paper and a pencil. He/she is given five minutes of preparation time for a fictive job interview. At this point, the test person is uninformed about details as well as the duration of the test. After the preparation, the committee prompts the test person to begin with their job application. The interviewers are instructed to keep a neutral face and voice, not to react to the various kinds of interaction attempts by the subject (smiling, laughing, initiation of a conversation etc.), and can only use a small repertoire of fixed phrases to communicate (see Appendix). After five minutes the test subject is told that they must complete another task: counting backwards from 2043 in decrements of 17. This activity continues for another five minutes, after which the committee thanks the participant and asks them to leave the room. The subject is then guided back to their seat by the experimenter.

In general, response rates of a two- to threefold rise in salivary cortisol levels are reported in over 70% of test persons (Kudielka et al., 2009). In a meta-analysis of 208 studies by Dickerson & Kemeny (2004), this effect is primarily attributed to three factors, which in combination proved to be especially important for the induction of a HPA-axis cortisol response: *First*, administration of a motivated performance task (preferred combining free speech in front of an audience and an arithmetical task): *second*, the relative uncontrollability of the task outcome, and *third*, the presence of social evaluation (Dickerson & Kemeny, 2004). Overall the TSST has shown to be a highly reliable and effective method [8] to provoke a cortisol response of the HPA-axis under standardized experimental conditions (Dickerson & Kemeny, 2004; Foley & Kirschbaum, 2010; Kudielka et al., 2009).

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[8] The condition of uncontrollability (b) is met by giving the test persons no precise information about the length of the task ahead, no feedback towards one's performance from the committee, and a general reduction of interaction to short, standardized sentences. Social-evaluative threat (c) is realized in the TSST through committee, their introduction as schooled behavioral observation psychologist, and the fake recording of the procedure by a microphone and video camera.

4.4 Assessment of Stress

4.4.1 Early Stressful Life Events

The short form of the CTQ (Bernstein et al., 2003) was administered during the pre-appointment (4.1). The CTQ is a retrospective checklist of childhood maltreatment, which has a high internal consistency, reliability, and criterion validity (independent ratings by therapists) (Bernstein et al., 2003; Wingenfeld et al., 2010). It uses a five-level Likert scale (0 = never, 1 = rarely, 2 = sometimes, 3 = often, 4 = very often) and encompasses five factors (physical, sexual, and emotional abuse & physical and emotional neglect). Further, the CTQ is assisted good measurement invariance in clinical and healthy groups considerably differing in age, sex, ethnicity, psychopathology, and life experience (Bernstein et al., 2003). It is therefore an ideal instrument to measure diverse individuals and also capping a wide spectrum of early traumata. In addition, the CTQ has already been successfully used in G × E interaction- (Aguilera et al., 2009; Fisher et al., 2013; Grabe et al., 2012; Klauke et al., 2011; Klengel & Binder, 2013; Nugent et al., 2011; Power et al., 2013; Reinelt et al., 2013; Stein et al., 2008) and epigenetic research (Mehta et al., 2013). Participants were categorized as traumatized, according to the well-established CTQ cut-off scores: (emotional abuse ≥ 13, physical abuse ≥ 10, sexual abuse ≥ 8, emotional neglect ≥ 15, and physical neglect ≥ 10). Furthermore, individuals were grouped as none-, mild-, and severe traumatized by the use of the mild CTQ cutoff scores (emotional abuse ≥ 9, physical abuse ≥ 8, sexual abuse ≥ 6, emotional neglect ≥ 10, physical neglect ≥ 8).

4.4.2 Cortisol Analysis

To obtain the saliva for cortisol analysis, participants were instructed to chew on cotton rolls (Salivettes, Sarstedt, Nümbrecht, Germany) for approximately three minutes. These cotton swabs were then stored in plastic containers and kept in a fridge at -20°C. For subsequent cortisol analysis, samples were defrosted and centrifuged at 3000 rpm for three minutes. Salivary free cortisol levels were determined with the commercially available cheiluminescence-immunoassays (IBL, Hamburg, Germany) with intra- and inter-assay precision of 3.0% and 4.2%.

4.5 Genotyping of the 5-HTTLPR genotype and 5-HTTLPR/rs25531 mini haplotype

Genotyping of the 5-HTTLPR INDE length polymorphism and the 5-HTTLPR/rs25531 mini haplotype was conducted according to a previously published protocol (Alexander et al., 2009). DNA was extracted from EDTA whole blood using a standard commercial extraction kit (High Pure PCR Template Preparation Kit; Roche) in a MagNA Puce LC System (Roche). The sample was furthermore genotyped for the rs25531 SNP, diverting the L allele into a low transcribing L_G and a high transcribing L_A variant. Individuals were genotyped according to the following protocol (Alexander et al., 2009).

A standard commercial extraction kit (High Pure PCR Template Preparation Kit; Roche, Mannheim, Germany) was used to collect DNA from buccal cells in a MagNA Pure LC System (Roche). The 5-HTTLPRgenotype was determined by polymerase chain reaction (PCR) and gel electrophoresis. DNA amplification reactions were conducted using a Mastercycler$_1$ ep (Eppendorf, Hamburg, Germany) as described below. Using the QIAGEN Multiplex PCR Master Mix (Qiagen, Germany), about 50 ng of genomic template DNA were amplified and 0.2 nM of the forward (5_0-TCC TCC GCT TTG GCG CCT CTT CC-3_0) and reverse (5_0-TGG GGG TTG CAG GGG AGA TCC TG-3_0) primer (TIB MOLBIOL, Berlin, Germany). Reactions were performed in a over all volume of 20 ml. Thermal cycling was carried out in a 5 min initial denaturation phase at 95 8C followed by 38 cycles of 94 8C (45 s), 59.5 8C (45 s) and 72 8C (45 s) each with a final extension step of 3 min at 72 8C. To analyze the rs25531 mini haplotype, 9 ml of PCR products were digested by MspI in a 20 ml reaction assay containing 1_ NEBuffer and 1_ BSA at 37 8C for 4 h. In the end, 12 ml of the restriction enzyme assay solution was segregated using gel electrophoresis on a 2% agarose-gel in TBE (160 V, 60 min) and visualized by ethidiumbromide.

4.6 *SLC6A4* Methylation Status

Bisulfite Pyrosequencing:

The quantitative methylation analysis of the 83 CpG sites in 799-bp *SLC6A4* promoter-associated CpG island (*SLC6A4* methylation) was conducted by Varionostic GmbH (Ulm, Germany), following the protocol as detailed below (Wankerl et al., 2014). EDTA

whole blood was used to extract DNA and was bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research, Range, CA, USA). Subsequent pyrosequencing was conducted using the Q24/ID System. Strict quality controls, which had to be passed by at least 90% of the examined 83 CpG sites led to the exclusion of 14 individuals. Mean methylation levels across the entire CpG island where therefore calculated for the remaining N=186 subjects. Subsequent analyses involving the *SLC6A4* methylation were conducted using this subsample.
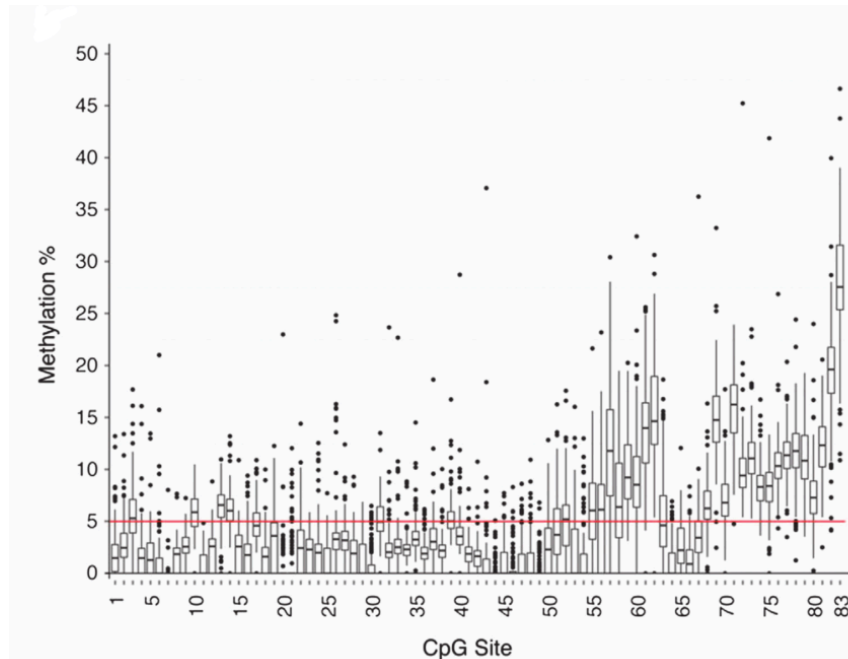
Figure 7 DNA methylation Boxplot diagram of DNA methylation levels of the 83 investigated CpG sites (Wankerl et al., 2014). The horizontal (red) line shows the detection limits.

SUPPLEMENT 2: Bisulfite Pyrosequencing Protocol

The EZ DNA Methylation Gold Kit (Zymo Research, Range, CA, USA) was used for bisulfite treating the genomic DNA and to generate 3 amplicons. The PCR protocols were carried out as follow: fragments 5HTT_P1 and 5HTT_P2 : HotStarTaq polymerase (Qiagen, Hilden, Germany), 95°C 15', 49x (95°C 35", 57°C 35", 72°C 35"), 72°C 5'; fragment 5HTT_P3: HotStarTaq polymerase (Qiagen, Hilden, Germany) 95°C 15', 49x (95°C 35", 52°C 35", 72°C 35"); 72°C 5'. Since pyrosequencing is most reliable in short reads, 10 sequencing primers were used to cover all 83 CpG sites of the 799 bp CpG island in the *SLC6A4* promoter-associated region. The sample was prepared by standard procedures using the Vacuum Prep Tool. 12-15µl PCR product was immobilized to 2µl Streptavidin Sepharose™ HP beads (GE Healthcare) followed by annealing to 0.8-1.0µl sequencing

primer (5µM) for 2' at 80°C. Amplicon and sequencing primers are depicted in Table 1 below.

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	Amplicon Primer name	Amplicon Primer Sequence 5´-3´	Seqencing Primer Name	Seqencing Primer Sequence 5´-3´
Amplicon 1 (CpG sites 1-13)	5HTT_P1-F	ggg ttt tta agt tga gtt tat at	5HTT_P1-S1 (CpG 1-7)	ttt agt agg tta gtt aga taa a
	5HTT_P1-R	Biotin- cta act ttc cta ctc ttt aac tt	5HTT_P1-S2 (CpG 8-13)	gag tag att ttt gtg tg
Amplicon 2 (CpG sites 14-42)	5HTT_P2-F	aag agt agg aaa gtt agg a	5HTT_P2-S1 (CpG 14-22)	gta gga aag tta gga ttt
	5HTT_P2-R	Biotin- ccc tca cat aat cta atc t	5HTT_P2-S2 (CpG 23-32)	ttt tgg ttt tgg ggt
			5HTT_P2-S3 (CpG 32-42)	ttg gag aga gta att tta
Amplicon 3 (CpG sites 43-83)	5HTT_P3-F	ggg gaa gta tta agt tta t	5HTT_P3-S1 (CpG 43-57)	att tag aga tta gat tat gtg
	5HTT_P3-R	Biotin- ccc cta caa caa taa aca	5HTT_P3-S2 (CpG 58-64)	agg tta gtt agt ttg ttt ag
			5HTT_P3-S3 (CpG 65-71)	att taa gtt ttt ttt tag at
			5HTT_P3-S4 (CpG 72-77)	agg aga gga ggt gta t
			5HTT_P3-S5 (CpG 78-83)	tta gta aga gtt aga gtt gaa

Table 1 Supplement 2 - Table. Amplicon and Sequencing Primers used for bisulfite pyrosequencing of the *SLC6A4* promotor-associated CpG island. All primers refer to bisulfite treated DNA.

4.7 Statistical Analysis

Statistical analyses were conducted using SPSS (Version 22.0.0.0, Chicago, IBM) for Macintosh. All tests were two-tailed and alpha level was set at 0.05. Chi-Square tests indicated no deviation from Hardy-Weinberg equilibrium for the bi-allelic ($\chi^2_{(1)}$ = 0.86, p = 0.35) and tri-allelic ($\chi^2_{(3)}$ = 5.73, p = 0.13) 5-HTTLPRgenotype. To be able to use the repeatedly measured cortisol levels in correlation analysis, as outcome variable in analysis of variance (ANOVA), and t-test designs, the area under the curve (AUC) for cortisol increase (AUC_i) (with respect to the first measurement point before the TSST) were calculated according to the trapezoidal formula published by Simmons, Nelson, & Simonsohn (2011). Kolmogorov-Smirnoff test indicated a deviation from normality for cortisol values (all p values $\leq$ 0.01). Consequently, these data were natural log transformed and used in the repeated measurement design to meet the assumption of normality for the outcome variable. Cofounders of outcome variables were investigated with repeated measurement ANOVA, independent t-tests and Pearson correlation. In Subsequent analysis identified covariates were considered in analysis of covariance (ANCOVA).

	AUC_i (N=186)		SLC6A4 methylation (N=186)	
	Statistic	P	Statistic	P
Gender	$F_{1,184}$ = 8.01	**0.01**	$t_{1,184}$ = 1.96	**0.05**
Smoking status	$F_{6,1104}$ = 2.53	**0.02**	$t_{1,184}$ = 0.73	0.47
OC use[a]	$F_{1,88}$ = 4.49	**0.04**	$t_{1,88}$ = 1.21	0.23
Body Mass Index	r = 0.11	0.82	r = - 0.08	0.27
Age	r = 0.87	0.24	r = 0.15	**0.04**
Abbreviations: TSST, Trier Social Stress Test; [a] in female subsample				

Table 2 Covariates of cortisol reactivity to the TSST and mean SERT methylation.

Smoking status, BMI, and female contraceptive use did not influence *SLC6A4* methylation. In contrast, participant's age (r = 0.15, p = 0.04) and gender (t = -1.96, p = 0.05) were associated with *SLC6A4* methylation levels and were therefore included as a covariate in subsequent analysis.

Converging with previously published literature (Philibert et al., 2007), cortisol reactivity was found to be influenced by sex (gender: $F_{1,184}$ = 8.01, p = 0.01, η^2 = 0.04; gender*time: $F_{6,1104}$ = 19.57, p = 0.01, η^2 = 0.1), smoking status (smoking*time: $F_{6,1104}$ =

2.53, p = 0.02, $\eta^2 = 0.01$), and contraceptive use in female participants (contraceptive: $F_{1,88} = 4.49$, p = 0.04; contraceptive*time: $F_{6,528} = 8.27$, p = 0.01, $\eta^2 = 0.09$), but not BMI and age (see Tab. 1). Consequently, analyses involving cortisol measurements were repeated controlling for gender, smoking status, and contraceptive use.

	5-HTTLPRgenotype					CTQ		
	Total (n=186)	SS (n=28)	SL (n=96)	LL (n=62)	P	Trauma (n=27)	None (n=159)	P
Sex (%females)	90 (48.4%)	18 (20%)	38 (42.2%)	34 (37.8%)	0.033	13 (14.4%)	77 (85.6%)	0.979
Age (years)	23.81 ± 2.81	23.57 ± 3.01	24.05 ± 2.87	23.53 ± 2.77	0.483	23.89 ± 2.77	23.79 ± 2.88	0.872
BMI	22.33 ± 2.91	22.49 ± 2.46	22.34 ± 2.08	22.23 ± 2.25	0.871	21.94 ± 2.04	22.39 ± 2.21	0.323
Smoker (yes%)	62 (33.3%)	9 (14.5%)	30 (48.4%)	23 (37.1%)	0.741	12 (19.4%)	50 (80.6%)	0.185
OC (yes%)	49 (26.3%)	11 (22.4%)	18 (36.7%)	20 (40.8%)	0.509	5 (10.2%)	44 (89.8%)	0.211

	5-HTTLPR/ rs25531 mini haplotype					SERT methylation		
	Total (n=186)	SS (SS,SL$_G$,L$_G$L$_G$) (n=36)	SL (SL$_A$, L$_G$L$_A$) (n=104)	LL (L$_A$L$_A$) (n=46)	P	low	high	P
Sex (%females)	90 (48.4%)	18 (20%)	48 (53.5%)	24 (26.7%)	0.775	41 (45.6%)	49 (54.4%)	0.240
Age (years)	23.81 ± 2.86	23.75 ± 2.98	23.99 ± 2.88	23.43 ± 2.73	0.546	23.54 ± 2.64	24.08 ± 3.04	0.201
BMI	22.33 ± 2.19	22.53 ± 2.52	22.21 ± 2.00	22.43 ± 2.35	0.696	22.54 ± 2.24	22.11 ± 2.12	0.184
Smoker (yes%)	62 (33.3%)	10 (16.1%)	37 (59.7%)	15 (24.2%)	0.689	32 (51.6%)	30 (48.4%)	0.756
OC (yes%)	49 (26.3%)	11 (22.4%)	24 (49%)	14 (28.6%)	0.653	20 (40.8%)	29 (59.2%)	0.324

Abbreviation: BMI, Body Mass Index; OC, Oral contraceptive; CTQ, Childhood Trauma Questionnaire

Table 3 Initial group comparisons

Initial group comparisons (see Tab. 2) were conducted using chi-square tests and ANOVAs. Traumatized vs. non-traumatized individuals showed no deviation from the expected 5-HTTLPR alleles distribution. Further, demographic variables were evenly distributed in the traumatized and non-traumatized population (all p values ≥ 0.19). Bi-allelic and tri-allelic 5-HTTLPRgenotype groups did not differ regarding age, smoking status, and contraceptive use, but showed a deviation from the expected distribution in male and female participants for the bi-allelic ($\chi^2_{(2)} = 6.85$ p = 0.03), but not tri allelic classification ($\chi^2_{(5)} = 0.51$, p = 0.78). Consequently analysis including the bi-allelic 5-HTTLPRgenotype, were additionally repeated with gender as a covariate.

5. Results

5.1 Main Effect of ESLE on *SLC6A4* methylation

In this section, an effect of early traumatization on *SLC6A4* methylation has been investigated.

Pearson's correlation did not show an association between total CTQ score and *SLC6A4* methylation (r = 0.52, p = 0.5). ANOVAs were conducted to compare traumatized (mean: 5.03, sd = 0.93) against non-traumatized individuals (mean: 4.91, sd = 1.09) according to their methylation status. Analyses revealed no difference between these groups in *SLC6A4* methylation levels ($F_{1,184}$ = 0.32, p = 0.57).

In a next step, ANCOVAs with the covariates gender, age, and contraceptive use (see Tab. 1) were calculated. Incorporating these confounders did neither reveal a significant association between trauma and *SLC6A4* methylation ($F_{1,181}$ = 0.24, p = 0.62).

In summary, the present study cannot confirm an association between exposures to traumatic events and *SLC6A4* methylation.

5.2 Main Effect of 5-HTTLPRgenotype on *SLC6A4* methylation

In this paragraph an effect of the 5-HTTLPR on *SLC6A4* methylation has been examined.

ANOVAs with *SLC6A4* methylation as outcome variable were conducted for the low (SS, SL_G, L_GL_G), intermediate (SL/ SL_A, L_GL_A) and high (LL/L_AL_A) transcribing 5-HTTLPRgenotypes (bi-allele/tri-allele). Additionally, S- and L allele dominant models were calculated.

ANOVAs did not show an association between the 5-HTTLPRgenotype (bi: $F_{2,183}$ = 0.12, p = 0.89; tri: $F_{2,183}$ = 0.16, p = 0.85) and *SLC6A4* methylation. Likewise showed the S- and L allele-dominant models no significant associations (all p values ≥ 0.59).

Repeating these procedures including the covariates gender, age, and contraceptive use, ANCOVAs similarly revealed no effect of the 5-HTTLPRgenotype on mean methylation levels (all p values ≥ 0.56).

In conclusion, this study does not support an effect of 5-HTTLPRgenotype on mean *SLC6A4* promoter region by bi- or tri-allelic (and S-, or L allele dominant) models.

5.3 Interaction effect of ESLE and 5-HTTLPRgenotype on *SLC6A4* methylation

In this section the possible interaction effect of early traumatic experiences and the 5-HTTLPRgenotype on *SLC6A4* methylation has been investigated.

Crossing the 5-HTTLPRgenotype groups and traumatized vs. non-traumatized individuals for the planned 2x2 factorial ANOVA design led to substantial differences in cell size distribution, including very small group sizes (e.g. n = 2 for traumatized S/S allele carriers). Therefore, CTQ scores were regrouped into non-traumatized, mild-traumatized, and severe-traumatized participants (CTQ mild cut of scores see 4.4.1). This newly computed trauma-variables were then crossed with the 5-HTTLPRgenotype groups. The resulting cell sizes were satisfying with all n > 20 for the S allele dominant model of the 5-HTTLPRgenotype and 5-HTTLPR/rs25531 mini haplotype (Caspi et al., 2003; Karg et al., 2011).

Thus, 2x2 factorial ANOVA was conducted based on either the 5-HTTLPRgenotype or rs25531 using the S allele dominant model and non-traumatized vs. mild- and severe-traumatized individuals.

Factorial ANOVA did not reveal a significant interaction effect of bi-allelic ($F_{1,182} = 0.420$, p = 0.518) or tri-allelic ($F_{1,182} = 0.011$, p = 0.917) 5-HTTLPRgenotype S allele carriers in interaction with early traumata on *SLC6A4* methylation.

Analyses were repeated incorporating gender, age, and contraceptive using ANCOVAs. Calculations did not show an effect of the 5-HTTLPR S allele (bi-allelic: $F_{1,179} = 0.10$, p = 0.752; tri-allelic: $F_{1,179} = 0.379$, p = 0.539), or trauma exposure (bi-allelic: $F_{1,179} = 0.024$, p = 0.877; tri-allelic: $F_{1,179} = 0.115$, p = 0.734). Likewise, did an ANCOVA reveal no significant interaction effect between 5-HTTLPRgenotype and early trauma on *SLC6A4* methylation (bi-allelic: $F_{1,179} = 0.288$, p = 0.592; tri-allelic: $F_{1,179} = 0.001$, p = 0.991).

In summary, there was no G × E interaction effect of the 5-HTTLPRgenotype and trauma exposure on *SLC6A4* methylation detected in this study.

5.4 Main Effect of *SLC6A4* methylation on cortisol reactivity

In this paragraph, an association between *SLC6A4* methylation and cortisol secretion in response to the TSST was explored.

Table 3 (p. 56) shows the measured cortisol salivary levels dependent on *SLC6A4* methylation levels, which were grouped by median split into a 'high' and 'low' methylated group.

Regarding the influence of 'high' versus 'low' *SLC6A4* methylation on cortisol reactivity, repeated measurement ANCOVA did not reveal an effect of DNA methylation on the HPA-axis stress response (*SLC6A4*: $F_{1,183} = 0.041$, p = 0.839; *SLC6A4* * time: $F_{6,1098} = 0.359$, p = 0.905).

To be able to calculate Pearson correlation coefficient using cortisol reactivity, the AUC_i was used (see 4.7). The procedure was repeated for total cortisol output using the AUC_g (see 4.7).

Bivariate Pearson correlation coefficient did no reveal a significant association between *SLC6A4* mean methylation and AUC_i (r = - 0.014, p = 0.844), respectively AUC_g (r = - 0.015, p = 0.844).

Thus, the data in the present study does not support an influence of *SLC6A4* methylation on HPA-axis reactivity to the TSST.

5.5 Main Effect of the 5-HTTLPRgenotype on cortisol reactivity

The TSST induced a significant salivary cortisol reaction in the overall sample ($F_{6,1110} = 192.566$, p= 0.001, $\eta^2 = 0.51$). Cortisol output dependent on 5-HTTLPRgenotype (respectively 5-HTTLPR#/rs25531 mini haplotype) is displayed in table 4 (p. 60). Mixed design repeated measurement ANCOVA revealed no main effect of the 5-HTTLPRgenotype (genotype: $F_{2,182} = 1.175$, p = 0.311; genotype * time: $F_{12,1092} = 1.531$, p = 0.107) on measured cortisol secretion. Similarly, the S- (genotype: $F_{1,183} = 2.357$, p = 0.126; genotype * time: $F_{6,1098} = 1.623$, p = 0.137) and L allele dominant models (genotype: $F_{1,183} = 0.151$, p =

.698; genotype * time: $F_{6,1098} = 1.148$, p = .332) were found to have no significant effect on cortisol reaction to the TSST.

Repeating analyses for the 5-HTTLPR/rs25531 mini haplotype showed also no significant effects (genotype: $F_{2,182} = .959$, p = 0.385; genotype * time: $F_{12,1092} = 1.271$, p = 0.230). Likewise, showed the S- (genotype: $F_{1,183} = 1.165$, p = .282; genotype * time: $F_{2.379,435.428} = 1.392$, p = 0.249) and L allele-dominant models (genotype: $F_{1,183} = 1.297$ p = 0.256; genotype * time: $F_{2.378,435.227} = 1.481$, p = .226) no significant effects.

Repeating these procedures by incorporating gender, smoking status, and contraceptive use as covariates, did not reveal any significant effect of the 5-HTTLPRgenotype on cortisol reactivity.

Cortisol (nmol/l)	Total	5-HTTLPRgenotype (rs25531 mini haplotype) (N=186)			SLC6A4 methylation (N=168)	
		SS (SS,SL$_G$,L$_G$L$_G$)	SL (SL$_A$, L$_G$L$_A$)	LL (L$_A$L$_A$)	Low	High
Baseline	10.21 ± 0.04	11.22 ± 0.10 (10.81 ± 0.09)	10.08 ± 0.05 (09.81 ± 0.05)	09.96 ± 0.07 (10.66 ± 0.08)	10.45 ± 0.06	09.97 ± 0.06
Post TSST	15.40 ± 0.49	15.87 ± 1.13 (15.91 ± 1.00)	16.09 ± 0.61 (15.41 ± 0.59)	14.11 ± 0.76 (14.97 ± 0.88)	16.63 ± 0.62	15.17 ± 0.62
+ 10 min	21.89 ± 0.88	22.29 ± 2.01 (23.42 ± 1.78)	23.38 ± 1.08 (22.28 ± 1.05)	19.41 ± 1.35 (19.83 ± 1.58)	22.00 ± 1.11	21.79 ± 1.11
+ 20 min	21.44 ± 0.98	23.05 ± 2.23 (23.81 ± 1.97)	22.66 ± 1.20 (21.62 ± 1.16)	18.81 ± 1.50 (19.81 ± 1.74)	21.81 ± 1.23	21.06 ± 1.23
+ 30 min	17.61 ± 0.79	19.49 ± 1.81 (19.56 ± 1.59)	18.27 ± 0.97 (17.64 ± 0.94)	15.73 ± 1.21 (16.02 ± 1.41)	17.73 ± 0.99	17.49 ± 0.99
+ 45 min	13.37 ± 0.52	14.71 ± 1.19 (14.42 ± 1.05)	13.59 ± 0.64 (13.09 ± 0.62)	12.43 ± 0.80 (13.18 ± 0.93)	13.22 ± 0.65	13.52 ± 0.65
+ 60 min	11.09 ± 0.40	12.57 ± 0.93 (12.51 ± 0.81)	11.12 ± 0.50 (10.82 ± 0.48)	10.36 ± 0.62 (15.57 ± 0.72)	10.89 ± 0.51	11.28 ± 0.51
Abbreviations: nmol/l, nanomol per liter						

Table 4 Mean salivary cortisol secretion levels before and after the Trier Social Stress Test as a function of 5-HTTLPRgenotype (and rs25331) and *SLC6A4* methylation levels.

5.6 Interaction effect of 5-HTTLPRgenotype and *SLC6A4* methylation on cortisol reactivity

To investigate hypothesis 2C, a mixed design repeated measurement ANCOVA was conducted. The seven recorded (4.3) cortisol values were used as within-subject factor and *SLC6A4* methylation and 5-HTTLPRgenotype as between subject factors. *SLC6A4* methylation levels were transformed into a 'high' and 'low' group by median split to incorporate them in a repeated measurement design. Cortisol saliva concentration at

measurement time point one (before the TSST) was used as baseline value to represent cortisol increase (4.7).

Analysis showed a significant interaction effect between the 5-HTTLPRgenotype and *SLC6A4* methylation on HPA-axis cortisol reactivity (genotype: $F_{2,179} = 3.657$, $p = 0.028$, $\eta^2 = 0.39$; genotype x time: $F_{12,1074} = 2590$, $p = 0.002$, $\eta^2 = 0.028$). Post hoc analyses for the 'low' and 'high' methylation group separately revealed a permitting role of 'low' *SLC6A4* methylation levels on the existence of the known 5-HTTLPRgenotype effect on cortisol reaction (2.1.3.3). The low methylation group displayed a significant effect of the 5-HTTLPRgenotype on measured cortisol levels (genotype: $F_{2,89} = 4.174$, $p = 0.019$, $\eta^2 = 0.086$; genotype x time: $F_{12,534} = 3.414$, $p = 0.001$, $\eta^2 = 0.071$). Cortisol secretion was dose-dependent, increasing with the occurrence of the S allele ($p = 0.01$).

In contrast, the 5-HTTLPRgenotype had no effect on cortisol response pattern in the 'high' methylated population (genotype: $F_{2,89} = 0.716$, $p = 0.492$; genotype x time: $F_{12,534} = 0.741$, $p = 0.711$).
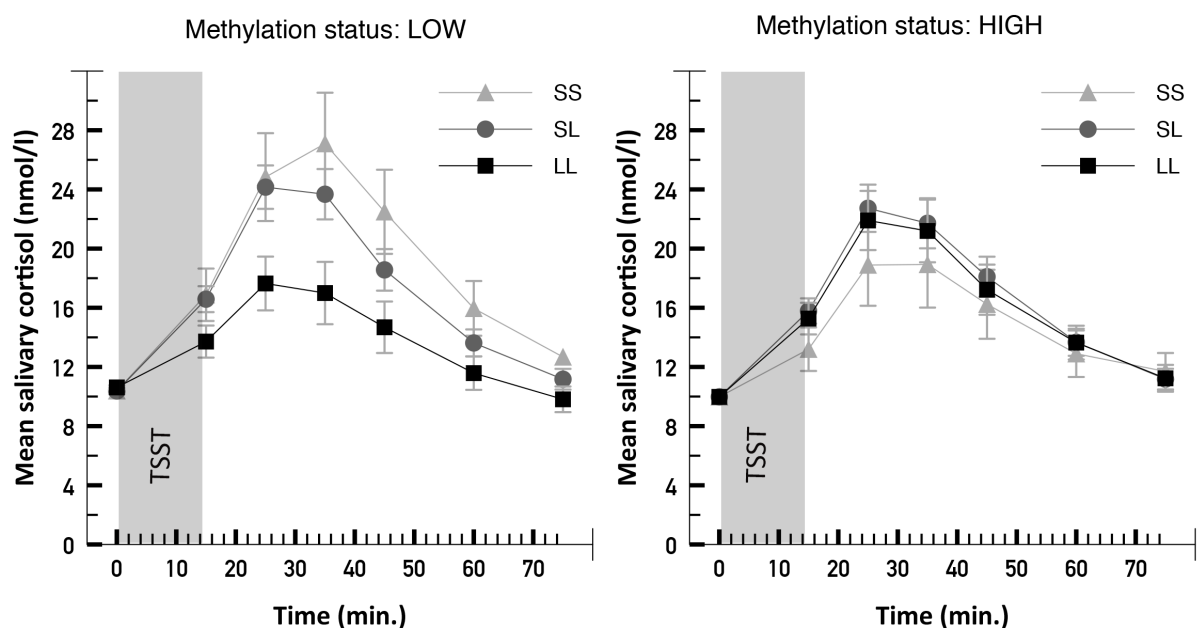
Figure 6 Result 5-HTTLPRgenotype Comparison of the 'low' and 'high' methylation group subdivided by the 5-HTTLPRgenotype allele combinations. The 'low' *SLC6A4* methylation group shows the established pattern of increased cortisol reactivity to the Trier Social Stress Test (TSST) associated with the S allele (left side). In contrast, 'high' methylation individuals are characterized by a uniform cortisol response across all genotypes (right side).

This relation of a 'permissive', respectively 'suppressive' influence of 'low', respectively 'high' *SLC6A4* methylation on the emergence of the established S allele effect on cortisol reactivity was also identified when reanalyzing the data for the rs25531 (Fig. 7). The 'low' methylation group expressed a significant dose-dependent effect of the S allele on increased

cortisol response to the TSST (genotype: $F_{2,89} = 3.969$, p = 0.022, $\eta^2 = 0.082$; genotype x time: $F_{12,534} = 2.857$, p 001, $\eta^2 = 0.60$), whereas the 'high' group did not (genotype: $F_{2,89} = 0.252$, p = 0.778; genotype x time: $F_{12,534} = 0.427$, p = 0.953).

Repeating these procedures by including gender, smoking status, and contraceptive use as covariates led to similar results, which confirms the robustness of the detected gene – epigenetic interaction (genotype (bi-allelic) x time: $F_{12, 1062} = 1.926$, p = 0.028, $\eta^2 = 0.021$; genotype (tri-allelic) x time: $F_{12,522} = 1.909$, p = 0.031, $\eta^2 = 0.042$).

In conclusion, this study could identify an interaction effect of *SLC6A4* methylation and the 5-HTTLPRgenotype on HPA-axis reactivity to the TSST. The nature of this relation is a suppressive effect of 'high' *SLC6A4* methylation on the 5-HTTLPRgenotype dependent cortisol response patterns to psychosocial stress. When *SLC6A4* methylation was 'low', S allele carriers experience a dose-dependent increase in HPA-axis cortisol secretion to the TSST.
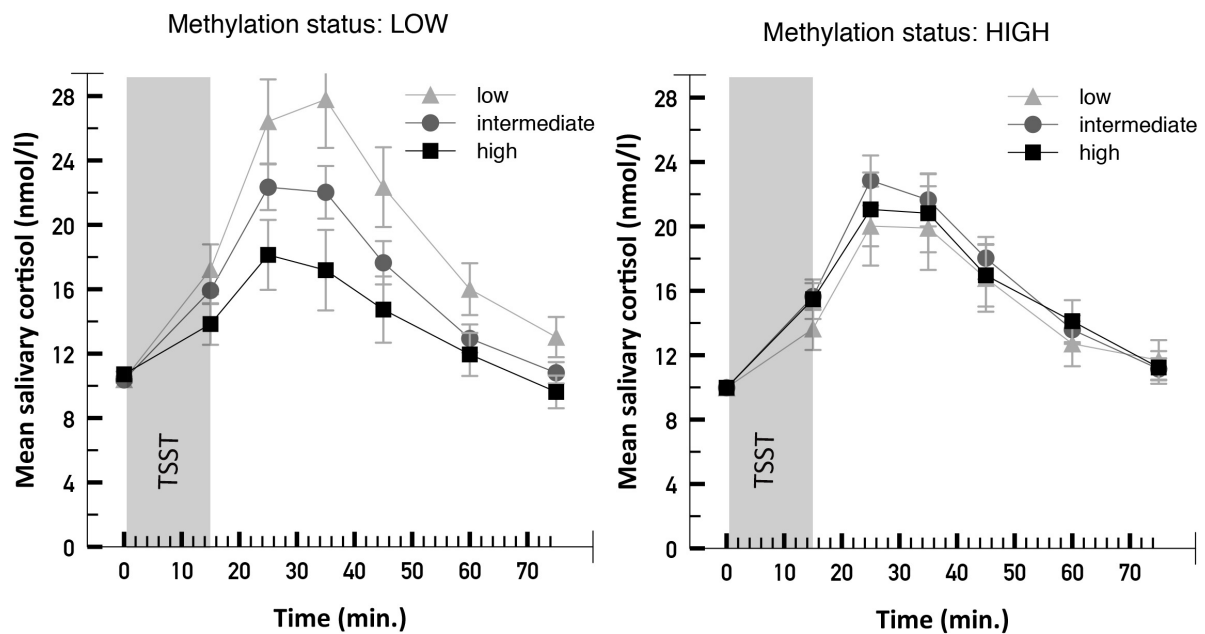

Figure 7 Result 5-HTTLPR/rs25531 mini haplotype Comparison of the 'high' and 'low' methylation group subdivided by the rs25531. The effects of *SLC6A4* methylation parallel those of the 5-HTTLPRgenotype. 'Low' methylation is associated with the established HPA-axis response pattern to stress, whilst 'high' methylation results in an uniform cortisol reactivity.

6. Discussion

The goal of this study was to elucidate the influence of early traumatization and the 5-HTTLPRgenotype on *SLC6A4* methylation. Moreover, the effect of *SLC6A4* methylation in the context of the 5-HTTLPRgenotype on HPA-axis reactivity to psychosocial stress was investigated. Now, the results of this study will be discussed, critically reviewed, and related to current findings in the field.

6.1 Interaction of genetic with epigenetic information on HPA-axis stress reactivity

The major finding of this study is a genetic - epigenetic interaction effect on HPA-axis reactivity to psychosocial stress (in the form of the TSST). This interaction effect between the 5-HTTLPRgenotype and relative 'low' *SLC6A4* methylation levels is characterized by on the S allele dose-dependent increase in cortisol output. In contrast, the 'high' methylation group experienced a uniform cortisol response pattern across all genotypes. The characteristics of this interaction were reproduced when repeating the procedure with the 5-HTTLPR/rs25531 mini haplotype.

Neither the 5-HTTLPRgenotype, nor mean methylation levels in the *SLC6A4* promoter-associated region showed a direct effect on cortisol stress reactivity. As for *SLC6A4* methylation, this result does not replicate the findings of Ouellet-Morin et al. (2013) of an effect of DNA methylation on cortisol reactivity. However, it should be kept in mind that Ouellet-Morin et al. (2013) could likewise not establish an association between mean methylation levels of the 12 investigated CpG sites and HPA-axis reactivity. Their positive finding was attributed to a single CpG dinucleotide and not mean methylation levels (Ouellet-Morin et al., 2013). Furthermore, the cortisol reactivity in 10 year old school children were investigated, which additionally reduces the comparability to this study (Ouellet-Morin et al., 2013).

Coming back to the significant interaction effect of this study, the explained variance increased from 0% to 7-9% when looking at the 'low' methylation group. In comparison showed the 'high' methylation group no association between 5-HTTLPRgenotype and HPA-

axis reactivity. This interaction effect might further explain why no main effect of 5-HTTLPRgenotype or *SLC6A4* methylation was detected when both 'high' and 'low' methylation groups are processed simultaneous. Consequently, inconsistencies and non-replications in the research of the 5-HTTLPRgenotype could be partially related to the hitherto ignored epigenetic profiles in the *SLC6A4* promoter-associated region.

From a molecular perspective, the attenuation of the S alleles effects on cortisol reactivity by increased *SLC6A4* methylation might seem counterintuitive. One might suspect increased methylation to amplify the effects of the 5-HTTLPR S allele, since increased methylation, as well as the S allele have been associated with reduced SERT mRNA transcription (Collier et al., 1996; Lesch et al., 1996; Philibert et al., 2007, 2008). Following this rationale the found suppression of the S alleles effect on HPA-axis reactivity is surprising. An interpretation that could explain this result is similar to that of the 'SSRI-paradox' discussed earlier (2.2.1.1): the supposed effects of reduced SERT mRNA transcription through increased methylation or by the influence of the S allele might differ between early development and later stages of life, respectively between in vivo and in vitro investigations. Additionally, the moment of *SLC6A4* methylation was not determined. This prevents an interpretation of potentially timing specific influences on mRNA transcription, which might have unique effects during different developmental phases.

Despite no effect of ESLE on *SLC6A4* methylation was detected, the finding of a genetic with epigenetic interaction on the HPA-axis stress response might still depict an adaption to environmental stimuli: *First*, an increased vulnerability to develop psychopathology has been found in response to environmental stressors in carriers of the short allele of 5-HTTLPRgenotype (Caspi et al., 2003; Karg et al., 2011). This in turn has been proposed to be conveyed by an increase in HPA-axis stress reactivity (Alexander et al., 2009, 2012; Miller et al., 2012). *Secondly*, since hyper-, as well as a hyporeactivity of the HPA-axis is associated with psychopathology (Chrousos, 2009), a balanced reaction to stressors (as seen in the 'high' methylation group) is regarded as an adaptive and healthy response (McEwen, 2007). *Thirdly*, there exists evidence that methylation in the *SLC6A4* promoter-associated region might serve as a protective mechanism. Increased levels of DNA methylation in this region have been linked with reduced mRNA transcription (Philibert et al., 2008) and might exert similar effects then AD drugs that target the 5-HT system. *Fourthly*, first findings point towards an increase in methylation in this region in response to ESLE (Beach et al., 2011, 2010; Kang et al., 2013; Ouellet-Morin et al., 2013; Vijayendran et al., 2012). The finding of a protective

role (balanced HPA-axis reactivity) of 'high' methylation in this study and the known malleability of DNA methylation by external stimuli suggests an adaption to the environment.

In intermediary summary, in the light of the current available data, it is conceivable that DNA methylation in the promoter-associated region of the *SLC6A4* could serve as a protective adjustment to early trauma, which in association with the stress sensitive S allele could otherwise lead to HPA-axis hyperreactivity (Alexander et al., 2009; Caspi et al., 2003; Karg et al., 2011). Although this study did not to identify a direct effect of ESLE on *SLC6A4* methylation, it could show a direct functional influence of epigenetic modification (in the form of DNA methylation) on HPA-axis reactivity. In the context of stress research, 'high' levels of methylation in this region seem to have an adaptive effect on the organisms stress response.

This rational is supported by a study from van IJzendoorn et al. (2010), who investigated the mean methylation over 71 CpG sites in a region of the *SLC6A4* promoter-associated region. They report that individuals homozygote for the S allele were at higher risk for unresolved loss or trauma, but only in combination with 'low' mean methylation levels (van IJzendoorn et al., 2010). In individuals with 'high' methylation levels, the S/S genotype was associated with less unresolved loss or trauma (van IJzendoorn et al., 2010). Furthermore, there exists evidence of an association between decreased *SLC6A4* methylation and depression: a study by Devlin et al. (2010) on prenatal adversity observed a reduction in *SLC6A4* promoter-associated region methylation in association with maternal depression, which is a known risk factor for the child to develop depression later in life (Hammen, 2005). These results point towards an decrease in DNA methylation in response to early life stress (Devlin et al., 2010). Finally, a notable feature of this study is the investigated population of young healthy adults, as these individuals might substantially differ from the clinical population. Especially in the light of an adaptive modulation hypothesis of DNA methylation in the *SLC6A4* promoter region, being healthy can be seen as a successful adaptation.

At this point it has to be noted that there exist results contradicting the above-elaborated reasoning. Studies by Olsson et al. (2010), Beach et al. (2011) and Kim et al. (2013) speak against a protective modulation of the 5-HTTLPRgenotype by increased *SLC6A4* methylation. Olsson et al. (2010) neither found an association between *SLC6A4* promoter methylation nor the 5-HTTLPRgenotype with depression, but instead found a joint effect of high methylation levels with the S allele to result in an increased risk for depression.

Moreover, Beach et al. (2011) report an increased risk for ASPD associated with heightened *SLC6A4* methylation in the context of the 5-HTTLPRgenotype S allele. Furthermore, Kim et al. (2013) report an association between DNA methylation in the *SLC6A4* promoter region with PSD and worsening of depression symptoms, which were more strongly pronounced in individuals homozygote for the S allele (though no direct genetic with epigenetic interaction on symptoms emerged). It should be kept in mind that the direction of increased, respectively decreased methylation and risk or adaption is unique to different psychopathologies.

Additionally, expanding the focus to studies not controlling for the 5-HTTLPRgenotype, but investigating the *SLC6A4* promoter-associated region, the results seem to contradict a protective role of increased methylation, and rather point towards an association with risk for MDD. Philibert et al. (2008) reported a trend association between increased *SLC6A4* methylation and the vulnerability to lifetime MDD, whereas Zhao et al. (2013) observed an 4.4 point increase in BDI scores linked to 10% increase in methylation levels. Furthermore, Kang et al. (2013) reported that increased *SLC6A4* promoter-associated region methylation is associated with depression in the family, heightened stress perception, and increased psychopathology.

If taken into account that an association between childhood adversity and increased methylation is one of the most consistent findings to date (Klengel et al., 2014), a protective function of epigenetic modulation as a reaction to these events is still imaginable. Even of in retrospective investigation an association between psychopathology and increased methylation is detected, this increase in methylation could be seen as an initial attempt to compensate for an unfavorable development in response to ESLE. It is therefore conceivable that we observe an association between heightened *SLC6A4* methylation levels and MDD, when in reality increased methylation has been an unsuccessful (or insufficient) adaption attempt during a certain period of life.

To summarize, there exists conflicting evidence for a protective or exposing effect of hyper-, respectively hypomethylation of the *SLC6A4* promoter-associated region. Final conclusions and the integration into the bigger picture of the association between mental disorders (such as MDD) and DNA methylation is at this point premature. Nevertheless, could this study identify a significant interaction effect between DNA methylation and the 5-HTTLPRgenotype on HPA-axis reactivity to psychosocial stress. If reproduced, this result can prove the functional relevance of epigenetic modifications in the *SLC6A4* promoter-associated region on HPA-axis stress reactivity.

Another central point of interest in this study was to elucidate the suggested contribution of epigenetic processes in reported G × E interaction effects between the 5-HTTLPRgenotype and ESLE on long term changes in HPA-axis reactivity (Brown & Harris, 2008). Although the present study could not identify a direct effect of early traumatization on mean *SLC6A4* methylation levels, it is not inconceivable that the observed genetic with epigenetic interaction on HPA-axis reactivity is a molecular mechanism of a G × E interaction. ESLE have been associated with increased DNA methylation in the *SLC6A4* promoter-associated region (2.2.2.2). Recalling that the present study did not register environmental factors other than ESLE that affect DNA methylation, the presented results coincide with an earlier study by Ouellet-Morin et al. (2008). In this investigation, Ouellet-Morin et al. (2008) researched the contribution of genetic and environmental factors on HPA-axis reactivity in 19-month-old twins in the context of family adversity (FA). In low FA children, similarities in cortisol reaction were determined by genetic factors, whereas differences arose due to unique environmental influences (Ouellet-Morin et al., 2008). Conflictingly, in high FA individuals, environmental factors solely accounted for the interindividual variance, but genetic factors had no longer an effect (Ouellet-Morin et al., 2008). These results, viewed as environmental conditions (stress due to FA) overwriting the genetic influence on HPA-axis reactivity, coincide with the observed effect of increased methylation levels on suppressing genotype related cortisol response patterns in this study. The finding of a unification effect through 'high' methylation on cortisol response patterns by 5-HTTLPRgenotype could be a potential molecular mechanism forming the basis for these observations. Support for an epigenetic contribution in such effects comes from a subsequent longitudinal monozygotic twin study by Ouellet-Morin et al. (2013). This study investigated the differences in HPA-axis reactivity and *SLC6A4* promoter-associated region methylation status between a bullied and non-bullied sibling (Ouellet-Morin et al., 2013). It was shown that cortisol secretion to stress was reduced in the bullied twin, which was additionally associated with increased *SLC6A4* methylation levels (Ouellet-Morin et al., 2013). Ouellet-Morin et al. (2013) hypothesized that *SLC64A* methylation might moderate the link between ESLE and an altered HPA-axis stress response. The results of this study support such an influence of DNA methylation on HPA-axis stress reactivity in interaction with the 5-HTTLRgenoype.

Again, there exist findings exists that contradict such an interpretation. In another study by Ouellet-Morin et al. (2009), the investigation of six-month-old infants revealed the opposite effect, when investigating morning cortisol levels under laboratory settings. In this

study, the genetic influence was pronounced under the high FA and had no contribution when FA was low (Ouellet-Morin et al., 2009). However, if individuals were measured at home, they exhibited the known pattern of a genetically influenced cortisol secretion. These contradictions underline the gap in our current knowledge about the direction, timing and context of epigenetic adjustments.

In conclusion, the present study proposes the inclusion of epigenetic data in the research of the 5-HTTLPRgenotype and psychiatric genetics. The interaction between mean *SLC6A4* methylation and the 5-HTTLPRgenotype increased the explained variance HPA-axis reactivity from 0% to 7-9% in the 'low' methylation group. The unification of cortisol response patterns in 'high' methylation individuals highlights how epigenetic modulation can dramatically alter genetic effects. The so far ignored layer of DNA methylation patterns might therefore help to solve the existing inconsistencies and small effect sizes apparent in the research of the 5-HTTLPRgenotype and the workings of the HPA-axis. Moreover, the inclusion of epigenetic data could improve clinical and neuroendocrine studies of the association between genetic predisposition and MDD. As has been argued (2.1.2.1), a big part of the depressive population contains a high prevalence of SLE, and ESLE have been found to increase risk to develop depression later in life. Recent research progressively establishes the connection between environmental stimuli (such as stress) and epigenetic modulation. In the light of the presented findings and the latest studies in the emerging field of behavioral epigenetic research, a contribution of DNA methylation in mental disorders is becoming increasingly probable. Hence, an inclusion of this layer of information in the field of clinical psychology is seen as an important endeavor.

6.2. Effects of Early Traumatization and the 5-HTTLPRgenotype on *SLC6A4* methylation

The present study did not identify a main effect of ESLE, the 5-HTTLPRgenotype or their interaction on *SLC6A4* methylation. Keeping in mind that a change in methylation state in the promoter-associated regions of the *SLC6A4* in the context of ESLE is the most consistent finding to date (2.2.2.2), the non-replication of this result was unexpected. There are several characteristics of this study and also of our state of knowledge and methodology that might help to explain this fact:

First, the timing of stressors might be a crucial factor mediating the impact of SLE. In the introduction, it has been illustrated that in animals critical developmental windows are open to adaption in response to environmental signals (2.1.3.2). In humans, neurological structures (such as the amygdala, hippocampus and PFC) develop in a differentiated temporal pattern (2.1.3.2). Moreover, the first two years of life constitute a phase of extreme neuronal conjunction and plasticity (Heim & Binder, 2012). By including SLE from several of these phases in one cohort (infancy, childhood, and early puberty) without distinguishing them by their temporal appearances, these events are equally weighed despite potential differences in their relevance: The loss of a parent might have different effects during infancy and at the age of 12. A stressor might even be crucial during a certain period of time, and irrelevant during another. For example Essex et al. (2013) found maternal stress to have a significant influence on children's methylation levels during infancy, but paternal stressors during preschool years. This shows that stressors might exert their effects during specific developmental periods and should be analyzed according to their temporal appearance.

An ideal approach would be to conduct longitudinal studies that investigate the methylation state of a gene of interest in the context of SLE measures. This kind of data could supply critical information on what kind of stressor influences methylation during which period and its direction.

Secondly, traumatic events after the 13th year of life have not been recorded in this study and could have confounded the measurement of *SLC6A4* methylation. Puberty is another period of increased plasticity and adaption (2.1.3.2) and SLE during that period might have further influenced methylation profiles.

Thirdly, the type of stressor might have differentiated influences on DNA methylation. Research has not yet elucidated the role of different trauma types on methylation. Sexual

abuse by a caretaker might exert a different effect than witnessing a natural disaster. Though scarce, there exists initial support for this assumption. In a genome-wide study investigating PTSD patients with and without childhood maltreatment, Mehta et al. (2013) reported a twelvefold increase in differentially expressed transcripts when early adversity had been encountered. Importantly, these transcripts were associated with different DNA methylation profiles and the authors remark that the mechanisms leading to a change in mRNA transcription are stressor type dependent (Mehta et al., 2013). Additional support for this idea stems from the aforementioned study by Essex et al. (2013), which hints at the different qualities of stressors (in this case maternal vs. paternal stress). Moreover, varying characteristics of stress appear to dissimilarly influence the effects on HPA-axis functioning (Booij et al., 2013). If DNA methylation is the mediator of this association, it should also differentially manifest itself in stressor dependent methylation profiles. The inclusive approach taken by this study might have therefore confounded stressor types that have different directional effects. It is also imaginable that simply the 'wrong' kinds of stressors have been investigated.

Fourthly, as has been mentioned earlier (2.1.2), the retrospective assessment of live events has several difficulties attached to it. For example, the operationalization of early traumata might be concealed by childhood amnesia (Hayne, 2004). Childhood amnesia refers to the inability to retrieve the earliest memories before approximately the third year of life (Hayne, 2004). It is a period of high plasticity and the unfolding of cognitive, emotional, and physical faculties (Hayne, 2004), which are driven by rapid brain development (2.1.3.2). This is mainly through an increase in neural connectivity (Hayne, 2004). Failure to record critical events during this shapeable period could have significantly distorted the investigated association between ESLE and *SLC6A4* methylation. Furthermore is the effect of recall bias influencing the accuracy of retrospective reports (Hardt & Rutter, 2004). Skepticism is further fueled considering the influence of GC on the hippocampus (2.1.3.2), a central structure in memory (Kim & Diamond, 2002). Excessive stress is associated with profound amnesia (Chu & Frey, 1999) and long term stress exposure with memory impairment and a 14% decrease in hippocampal volume (Chu & Frey, 1999). Additionally, memory is susceptible to suggestion, which can lead to the formation of pseudo memories (Hardt & Rutter, 2004). And finally, rates of 19% - 62% of amnesia for childhood sexual abuse are reported in the clinical population (Chu & Frey, 1999).

These considerations should be taken seriously and might have played a part in the results of the present study. However, precautions have been integrated and one should not

overestimate these effects (Hardt & Rutter, 2004). The sample consisted of young adults, making long-term effects of stress and age related problems with memory unlikely. Furthermore, healthy individuals were investigated and intensively screened for psychopathology (4.1), reducing the influence of mood- and disorder caused retrieval problems. And even though caution of a final conclusion about the validity of retrospective investigated life events is appropriate, "the bulk of memory research actually supports the accuracy of memory for the central components of significant events" (Chu & Frey, 1999, p. 750). Hardt & Rutter (2004) conclude in a review of the literature that reports about abuse and neglect are likely to be correct, if they can be meaningfully operationalized.

A suggested approach to further improve these potential imprecisions would be the use of objective data. Social insurance, hospital or welfare data could be used for information on accidents, violence, and abuse. Likewise are cohorts that are exposed to grand scale events of interest. Examples would be child soldiers in Uganda, witnesses of 9/11 or natural catastrophes.

Fifthly, this study did not include the information of prenatal environmental influences. The fetal origin/programming hypothesis locates the initial starting point for a variety of diseases during fetal development (de Boo & Harding, 2006). The basic idea is that the fetus adapts in advance to its likely future environment (de Boo & Harding, 2006). The source of information accessible to the developing child is in this case the microcosm of the maternal organism. The so far identified major factors are nutrients and glucocorticoid exposure (Field & Diego, 2008; Martini, Knappe, Beesdo-Baum, Lieb, & Wittchen, 2010; Robinson et al., 2008). As has been argued (2.1.1), is the glucocorticoid stress response closely linked to psychological processes and altered in various psychiatric diseases (2.1.2.1, 2.1.2.2). During pregnancy, clinical states of depression and anxiety are a common phenomenon in women (Martini et al., 2010) and are associated with a greater risk for the child to develop psychological problems later in life (Robinson et al., 2008). A suggested molecular mechanism mediating these alterations in response to environmental cues are epigenetic modifications (Conradt, Lester, Appleton, Armstrong, & Marsit, 2013; Hompes et al., 2013; Oberlander et al., 2014). In support of this hypothesis are recent studies linking maternal psychological states (Conradt et al., 2013; Hompes et al., 2013; Oberlander et al., 2014) and prenatal smoking (Flom et al., 2011; Toledo-Rodriguez et al., 2010) to altered offspring methylation levels. These studies could report a link between maternal anxiety (Mulligan, D'Errico, Stees, & Hughes, 2012), depression (Conradt et al., 2013; Oberlander et al., 2014) and extreme stress (Mulligan, D'Errico, Stees, & Hughes, 2012) with increased *GR* gene methylation.

Importantly, Devlin et al. (2010) reported an association between maternal depression and newborn *SLC6A4* methylation, emphasizing the possibility of prenatal modification at this gene of interest.

Despite the first wave of studies of the effect ESLE on *SLC6A4* methylation did not investigate prenatal factors, subsequent future investigations should include data on this sensitive developmental period. The plasticity of the epigenome after the earliest impregnation is central to the idea of epigenetic behaviorism and needs to be elucidated further.

Sixthly, protective or resilience factors could have influenced DNA methylation levels in the *SLC6A4* promoter-associated region. In a nutshell: if epigenetic modulations are in part caused by of traumatic experiences, they could similarly by influence by salutary events (Bowes & Jaffee, 2013; Homberg & Lesch, 2011; Masten, 2001). Following the same argumentative pathways for curative factors that have been used for the impact of stressful experiences, it becomes conceivable that salutary factors might be involved in the shaping of the epigenome.

Rodent early life manipulation studies show that maternal social behavior (in the form of licking and grooming) is able to influence DNA methylation (Champagne & Curley, 2005). A study investigating the effect of social deprivation on the HPA-axis observed that the resulting changes are reversible by later exposure to a socially enriched environment (Bredy, Humpartzoomian, Cain, & Meaney, 2003). Likewise, can the programming effects of prenatal stress on the HPA-axis be reversed by postnatal manipulations (overview see: Francis et al., 2002). Furthermore, cognitive deficits caused by low maternal care could be compensated by later environmental enrichment (Kawachi & Berkman, 2001). These observations from rodent studies convincingly show that social interaction can buffer, or at least influence the developmental effects of ESLE. Additionally, protective behavior can become a stable trait in the offspring and is thus transmitted to the next generation (Kawachi & Berkman, 2001). Naturally, there must be a biological mechanism conveying these effects.

In our current understanding, epigenetic mechanisms convey the molecular basis of such dynamic and at the same time stable traits that result from the interplay of genetic predisposition and environmental context.

Moving on from rodents to humans, the crucial role of social feedback in our development is highlighted by attachment theory (Oerter & Montada, 2002). In general, an association between mental health and social bonds has been established, whereas isolation is linked to an absence of well-being (Hjemdal, Friborg, Stiles, Rosenvinge, & Martinussen,

2006; Kawachi & Berkman, 2001; Kessler et al., 1985; Zolkoski & Bullock, 2012). Positive social experiences seem likewise able to 'buffer' the effects of stressful and traumatic events, especially during early developmental phases (Cohen & Hoberman, 1983; Francis, Diorio, Plotsky, & Meaney, 2002). In support of the 'buffering-hypothesis'[9] (Hjemdal, Friborg, Stiles, Rosenvinge, & Martinussen, 2006), die Kaufman et al. (2004) find a significant interaction effect between resilience scores and SLE. The increased presence of these factors prevented the pathogenic effects of SLE, but had no effect on mental health in the absence of adversity (Kaufman et al., 2004). Furthermore, a G × E interaction between the 5-HTTLPRgenotype and the history of child maltreatment and/or social support was observed (Kaufman et al., 2004). The S/S genotype and a past of maltreatment was associated with risk for depression (Kaufman et al., 2004). Significantly, if social support was available, maltreated children had similar depression scores as children without abuse experiences (Kaufman et al., 2004). The authors continue to state that the quality, but also the availability of social support is one of the central environmental variables to promote resilience, even in the face of vulnerability genotypes (Kaufman et al., 2004). This finding echoes the literature on resilience (Afifi & MacMillan, 2011; Cohen & Hoberman, 1983; Kessler et al., 1985; Ungar, 2013; Zolkoski & Bullock, 2012).

Similar to SLE, biological pathways for these effects have been investigated (Giesbrecht, Poole, Letourneau, Campbell, & Kaplan, 2013; Mikolajczak, Roy, Luminet, & de Timary, 2008; Uchino, Carlisle, Birmingham, & Vaughn, 2011). Giesbrecht et al. (2013) could, for example, show that social support during pregnancy moderated the association between distress and cortisol output. Furthermore, Mikolajczak et al. (2008) reported a significant negative association between resilience scores on anticipatory cortisol output to the TSST and a marginal reduction of stress hormone secretion during the procedure. Moreover, social support has been shown to decrease the ANS reaction to laboratory induced stress (Uchino et al., 2011).

Though scarcer than the literature on adverse experiences, the presented evidence supports the theory of an influence of protective factors on mental health through biological pathways, which should likewise be influenced by epigenetic programming (Bowes & Jaffee, 2013). To date there has been no investigation of the influence of these variables on epigenetic markers, but the presented accumulated evidence and the overall rationale of

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[9] Due to shortage of space, the interested reader is referred to the concepts of resilience (Luthar et al., 2000) and the 'buffering hypothesis' (Kawachi & Berkman, 2001).

behavioral epigenetics highly suggests this possibility. In conclusion, investigating protective and resilience factors in combination with SLE could enhance our understanding of G × E interactions and their facilitation by epigenetic modifications.

6.3 Future Studies

The preliminary results of this study show that DNA methylation is able to influence the effects of genes on complex upstream systems, in this case the HPA-axis stress response. In the context of contradictory results and small portions of explained variance in research of the 5-HTTLPRgenotype, additionally investigating the layer of epigenetic modulations in future studies could lead to improved results. It can be further expected that effects of DNA methylation in regulatory gene regions are not limited to the *SLC6A4* gene, and an inclusion of this new factor might advance the entire field of genetic research into mental disorder. Furthermore, could psychotherapeutically and pharmacological interventions be investigated regarding their effects on epigenetic profiles. This would not only increase our understanding of the working mechanisms of modern therapy approaches, but also open the door for an evidence-based evaluation grounded on objective molecular data. To achieve these goals, improvements to future studies are suggested.

First, subsequent studies should further investigate the specific effects of stressful experiences in different developmental phases. Important points in time are the prenatal period and the first and second year of life. As has been repeatedly argued, these phases are marked by growth and plasticity, therefore mapping this early phases should be priority. It is plausible that the organism undergoes its first stages of adaption to its expected environment during these time periods. Maternal stress, nutrition, and environmental pathogens are likely to have extended programming effects during pregnancy, which are potentially conveyed by epigenetic modulations. Additionally, newborns and infants are especially dependent on their social environment, and a disruption of these delicate ties is likely a major factor in the programming of the individual (e.g. HPA-axis reactivity). Cooperating with health institutions to combine longitudinal designs with routine examination could increase feasibility and reduce costs and dropout rates.

Secondly, the different impact of the various types of stressors should be further investigated. As has been argued, specific stressors (especially in interaction with developmental periods) could exert unique effects on epigenetic profiles. An interesting pattern in this context can be seen in the first wave of studies researching the association

between childhood trauma and *SLC6A4* methylation. Four out of five studies reporting an increase in DNA methylation in association with childhood adversity used the same type of stressor for their investigations (Beach et al., 2011, 2010; Ouellet-Morin et al., 2013; Vijayendran et al., 2012). Three studies only used two questions to asses sexual abuse (Beach et al., 2011, 2010; Vijayendran et al., 2012) and one only investigated a single type of stressor (bullying victimization) (Ouellet-Morin et al., 2013). Future studies could therefore either investigate sufficiently big samples to compare different types of stressors (ideal, but cost intensive) or focus on a single stressor.

Thirdly, basic research needs to further elucidate the exact mechanisms of the dynamics of methylation and demethylation. A penetrative understanding of these processes is needed to elaborate suited study design in behavioral epigenetic research. Time frames that are open to epigenetic plasticity could particularly help to better understand the impact of SLE in different periods of life. Animal manipulations might be necessary to broaden our understanding of these mechanisms.

Fourthly, the association between peripheral biomarkers (whole blood, T-cells, buccal cells etc.) and CNS methylation levels needs to be established. These associations might be gene specific and behavioral epigenetic research needs reliable information on this topic, since acquiring CNS tissue from living human subjects is currently not possible on a broad scale. Likewise, a unification of analysis methods and software in epigenetic research could help reduce potential measurement variability.

Fifthly, a potential advantage of objective measurement methods of stressful life events needs to be clarified. The retrospective assessment of life events is a contributor to inconsistencies in G × E studies. This should be similarly true in epigenetic research that includes SLE and ESLE. A counterargument is that the use of institutional data would also be accompanied by a massive reduction of accessibility to a major part of the population. Therefore is the use of epigenetic profiles as an objective indicator representing the sum of environmental influences throughout a lifetime another imaginable solution. Similarly, investigations into populations exposed to large-scale events could be a promising approach.

Finally, the author suggests including data of, and investigating the effects of protective factors and events in epigenetic research on mental disorders. Complex group dynamics,

prolonged rearing and the accompanied adaptability are trademarks of the human species. Social ties and working relationship are of pivotal importance for the survival of the individual and the group. As has been argued, it is plausible that nurturing relations and protective social factors could exert similar programming effects as SLE. Hitherto research even suggests that such variables can buffer the effects of traumatic and stressful experiences. Furthermore, effects on biological pathways (e.g. HPA-axis reactivity) have been observed in recent studies. It is therefore suggested to implement these variables in G × E interaction and epigenetic studies. The invested resources of applying an additional questionnaire might far outweigh the costs in the long run.

6.4 Limitations

There are several limitations to be considered in this study.

First, early traumatization has been retrospectively recorded. It has to be noted that despite the high quality of the applied questionnaire and the additional implementation of interview and screening methods that the retrospective acquisition of stressful experiences has been a contributor in qualitative deficits in G × E interaction research (Uher & McGuffin, 2008, 2010).

Secondly, peripheral tissue (whole blood) was used to determine *SLC6A4* methylation status, which may not generalize to the CNS. Despite this relevant concern (Olsson et al., 2010), provide recent studies evidence for a correlation between methylation levels measured across peripheral markers and brain tissue (Provençal et al., 2012). In mice (Lee et al., 2010) and rhesus monkeys (Provençal et al., 2012), a system-wide change in methylation profiles could be reported in response to environmental stressors. In a post mortem study in humans, Byun et al. (2009) found similar methylation profiles in 11 investigated tissues (including the brain). Furthermore, despite reporting overall greater intraindividual then interindividual tissue variability, Davies et al. (2012) also identified correlations between brain and blood methylation profiles of specific genes and DNA methylation at promoter CpG islands to be conserved across tissue. These results concur with findings by Klengel et al. (2014) of high similarity between CpG island methylation in somatic cells. Importantly, in case of *SLC6A4* methylation, Aberg & van den Oord (2011) found a correlation between blood *SLC6A4* promoter methylation with 5-HT brain synthesis using PET scans. Since DNA methylation in the promoter regions of genes is associated with transcriptional activity (2.2.2.1), is this a strong argument for the applicability of peripheral *SLC6A4* methylation measures in psychological research. Moreover is this point supported by the growing acknowledgment of peripheral measures in research into stress-related psychiatric disorders (Zhao et al., 2013).

Thirdly, whole blood was used for epigenetic analysis of the DNA. Despite the advantage of avoiding duplication procedures that potentially influence the epigenetic

signature (Contrada & Baum, 2011), the mixture of different cell types could be a confounding factor in the present study (de Kloet et al., 2006; Schüle et al., 2009).

Finally, the presented findings of an epigenetic modulation of the 5-HTTLPRgenotype' effects on HPA-axis reactivity to the TSST remains preliminary until further replication.

6.5 Summary

The results of this study do not confirm recent findings of an association between ESLE and DNA methylation in the promoter association region of *SLC6A4*. Furthermore, there was no G × E interaction effect between the 5-HTTLPRgenotype and early traumata on *SLC6A4* methylation profile detected.

Nonetheless, a significant genetic - epigenetic interaction effect between the 5-HTTLPRgenotype, respectively 5-HTTLPR/rs25531 and *SLC6A4* methylation on HPA-axis reactivity to the TSST could be revealed. The 'low' methylation group experienced a dose-dependent increase in cortisol secretion tributary to the presence of one, or two S alleles. In contrast, the 'high' methylation group exhibited a uniform response patterns across genotype groups. The consideration of epigenetic information resulted in an increase in explained variance from 0% to 7-9% in the 'low' methylation group.

Therefore it is suggested to include DNA methylation in modern research designs in psychiatric genetics and G × E interaction studies. The result of this study impressively demonstrates how epigenetic modifications are able to alter effects of genes on complex systems that are located downstream of genetic information. Moreover, the investigation of multiple layers of information (e.g. genetic, epigenetic, and endophenotypic) in one model appears to be a promising approach to tackle the complexity of behavioral epigenetic research. In the beginning of this thesis the connection between traumatic experiences, the 5-HTTLPRgenotype, and depression has been elaborated. The hitherto researched biological connection of these factors is the HPA-axis. The present study expands this model, showing the functional relevance of DNA methylation on the cortisol stress response. Therefore, considering epigenetic information might help to improve our understanding of the etiology of MDD and result in improved therapy and diagnostic approaches.

Nonetheless, the exciting implications of these results need to be replicated and further research has to elucidate the exact mechanisms of epigenetic modification.

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„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Solltedennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir."

8 Appendix

Telefon-Screening Epigenetikstudie

Datum: _____

Telefon-Screening

Inhalt:

- Telefon Screening

Versuchsleiter:

1

Name: _____

Vorname: _____

Telefon-Screening

> *„Guten Tag Frau/ Herr X, mein Name ist XXX und ich arbeite am Institut für Biologische Psychologie der TU Dresden. Sie hatten am …. (Datum) telefonisch/ per e-mail Ihren Namen und Ihre Kontaktdaten hinterlassen und damit Interesse an unserer biopsychologischen Untersuchung bekundet. Besteht dieses Interesse weiterhin?*
>
> *Haben Sie die Studieninformationen gelesen? Haben Sie noch Fragen zum Ablauf?*
>
> *Bevor ich Sie zu einem Untersuchungstermin in unser Institut einladen kann, muss ich überprüfen, ob Sie bestimmte Voraussetzungen erfüllen, um an der Untersuchung teilzunehmen. Dazu möchte ich Ihnen jetzt einige Fragen stellen, die Sie in der Regel mit „Ja" oder „Nein" oder in wenigen Worten beantworten können. Das Telefoninterview wird insgesamt etwa 10 Minuten dauern. Alle Informationen, die Sie uns geben, werden selbstverständlich vertraulich behandelt. Sind Sie damit einverstanden?"*

A. Erhebung Allgemeiner Daten

Name: _____   Tel.-Nr.: _____

Geschlecht: m   w   Geb.-datum: _____

Datum Kontakt: _____   (18-30) (ab 1993 – 1980)

> *Wie sind sie auf uns aufmerksam geworden?*

Was machen Sie beruflich?		
Waren Sie jemals in psychologischer, psychotherapeutischer oder psychiatrischer Behandlung? *Kein Ausschlusskriterium*	Nein ☐	Ja ☐
Falls Ja: Aus welchem Anlass? _____		
Wie oft haben Sie eine Psychotherapie begonnen? _____		
Wann haben Sie die letzte Psychotherapie begonnen? _____		
Wurden die Ziele in der Therapie erreicht?	Nein ☐	Ja ☐
Wie groß sind sie? _____m Wie viel wiegen sie? _____kg		
Haben Sie eine Haarlänge von mind. 3 cm? *Ausschlusskriterium*	Nein ☐	Ja ☐
Haben Sie Dreadlocks/Rastas, welche die Entnahme einer kleinen Haarsträhne verhindern würden? *Ausschlusskriterium*	Nein ☐	Ja ☐

B. Eignung

„Für unsere Studie müssen wir im Vorfeld prüfen, ob es Ausschlussgründe für diese Untersuchung gibt. Ich werde Ihnen nun einige Fragen diesbezüglich stellen.

Bitte antworten Sie die Fragen mit „Ja" oder „Nein".

Allgemeine Ausschlusskriterien		
Nehmen Sie regelmäßig Medikamente? (ZNS-Medikamente?) *Wenn ja, welche?* _____	ja	nein
Nehmen Sie regelmäßig Drogen zu sich? *Welche?:* _____	ja	nein
Nehmen sie Contraceptiva (Pille)?	ja	nein
Rauchen Sie?:	ja	nein
Sind ihre Eltern und Grosseltern deutscher Abstammung (Kaukasisch)?	ja	nein
Haben Sie akut eine körperliche Erkrankung? *Wenn ja, welche?* _____		
• Krankheiten im Ohrbereich (Schwerhörigkeit, Tinitus)	ja	nein
• Allergien/Überempfindlichkeitsreaktionen (Medikamente, Gräser, Heuschnupfen, Pollen, Latex)	ja	nein
• Autoimmunerkrankungen (Gastritis A, Neurodermitis, MS)	ja	nein
• Infektionserkrankungen (HIV, Hep., TBC)	ja	nein
• Schilddrüsenerkrankungen	ja	nein
• Herzerkrankungen (Angina pectoris, Herzinfarkt, Herzfehler)	ja	nein
• Kreislauf- und Gefäßerkrankungen (Durchblutungsstörung, zu hoher/niedriger Blutdruck, Thrombose)	ja	nein
• Lungen- und Atemwegserkrankungen (Tuberkulose, Bronchitis, Asthma) → *aktuelle Einnahme von Cortisonspray, Corticoidspray als Ausschluss*	ja	nein
• Lebererkrankungen (Hepatitis, Gelbsucht, Leberverfettung)	ja	nein
• Nieren- und Harnwegserkrankungen (Nierenbeckenentzündung, Nieren-/Blasensteine)	ja	nein
• Erkrankungen des Verdauungstraktes (Magenerkrankung, chronische Darmerkrankung)	ja	nein
• Stoffwechselerkrankungen (Diabetes m., Hypercholesterinämie)	ja	nein
• Erkrankungen des Skelettsystems/Muskelerkrankungen	ja	nein

• Bluterkrankungen (blaue Flecken ohne Anlass, Anämie)	ja	nein
• Tropenaufenthalt in den letzten 6 Monaten	ja	nein
• Impfungen die letzten 4 Wochen	ja	nein
Frauen: Besteht die Möglichkeit einer Schwangerschaft? **Ausschlusskriterium**	ja	nein
Raucher: Ist es für Sie für Sie problemlos möglich sein **über einen Zeitraum von 4 Stunden am Stück nicht zu rauchen**?	ja	nein
Haben Sie bereits in der Vergangenheit an einer vergleichbaren Studie teilgenommen? **Ausschlusskriterium**	ja	nein

Wir interessieren uns innerhalb der Studie außerdem für den Einfluss von frühen Kindheitserfahrungen auf die Hormonregulation im Erwachsenenalter. Ich würde daher im nachfolgenden einige solcher einschneidenden Ereignisse nennen und sie würden einfach mit JA antworten, falls sie eine oder mehrere solcher Erfahrung vor Ihrem 13. Lebensjahr gemacht haben. Ich werde dazu immer eine Gruppe von Ereignissen zusammenfassen, so dass Sie auch gar nicht genau nennen müssen, welches konkrete Ereignis jetzt auf sie zutreffen würde und Sie müssen auch keins dieser Ereignisse näher beschreiben oder näher ausführen. Bitte Antworten Sie einfach mit ja, wenn eines der folgenden Ereignisse auf Sie zutrifft:

Ist Ihnen vor dem 13ten Lebensjahr eines der nachfolgenden Ereignisse wiederfahren?
1. Sie haben eine Katastrophe (z.B. ein großes Feuer, ein Erdbeben) miterlebt
2. Sie haben einen schwere Unfall beobachtet (z.B. Autounfall, Arbeitsunfall)
3. Sie haben einen Raub, einen Überfall oder einen tätlichen Angriff beobachtet
4. Sie selbst wurden beraubt, körperlich angegriffen oder sexuell misshandelt von einer Person, die sie nicht kannten
5. Sie hatten selber einen schweren Unfall oder eine Unfallbedingte Verletzung
6. Sie selbst hatten eine schwere körperliche oder psychische Erkrankung

Ja   Nein   Anzahl:

Trifft eines der nachfolgenden Ereignisse auf ihr familiäres Umfeld vor dem 13. Lebensjahr zu?
1. Ein Familienglied von Ihnen musste ins Gefängnis
2. Sie wurden zu Pflegeeltern oder in ein Pflegeheim gegeben
3. Ihre Eltern haben sich getrennt, bzw. wurden geschieden
4. Sie hatten erhebliche Geldprobleme (z.B. nicht genug Geld für Essen)
5. Eine Ihnen nahestehende Person ist verstorben

Ja   Nein   Anzahl:

Trifft eine der nachfolgenden Bedingungen auf die Zeit vor Ihrem 13. Lebensjahr zu?

1. Sie haben Gewalt zwischen Familienmitgliedern beobachtet (z.B. Schläge)
2. Sie selbst wurden körperlich angegriffen oder misshandelt (darunter zählt jetzt sowohl körperlich auch sexuell) von einer Person, die sie kannten (z.B. Eltern)
3. Sie wurden physisch vernachlässigt (z.B. nicht gefüttert, alleine gelassen, wenn sie krank waren)
4. Sie wurden emotional vernachlässigt (z.B. ignoriert oder Ihnen wurde immer wieder gesagt, dass sie nicht gut genug sein).

Ja   Nein   Anzahl:

Gibt es irgendwelche traumatischen Erlebnisse, die Ihnen vor Ihrem 13. Lebensjahr widerfahren sind, die wir nicht berücksichtigt haben?

CTQ

Bitte lesen Sie sich die folgenden Aussagen zu Erlebnissen in der Kindheit durch. Kreuzen Sie dann bitte an, in welchem Maße Sie diesen Aussagen zustimmen.

nie	selten	manchmal	oft	sehr oft
0	1	2	3	4

	nie				sehr oft
	0	1	2	3	4

In meiner Kindheit …

1. … hatte ich nicht genug zu essen. ○ ○ ○ ○ ○

2. … wusste ich, dass sich jemand um mich sorgte und mich beschützte. ○ ○ ○ ○ ○

3. … bezeichneten mich Personen aus meiner Familie als „dumm", „faul" oder „hässlich". ○ ○ ○ ○ ○

4. … waren meine Eltern zu betrunken oder von anderen Drogen „high", um für die Familie zu sorgen. ○ ○ ○ ○ ○

5. … gab es jemanden in der Familie, der mir das Gefühl gab, wichtig und Besonders zu sein. ○ ○ ○ ○ ○

6. … musste ich dreckige Kleidung tragen. ○ ○ ○ ○ ○

7. … hatte ich das Gefühl, geliebt zu werden. ○ ○ ○ ○ ○

8. … glaubte ich, dass meine Eltern wünschten, ich wäre nie geboren. ○ ○ ○ ○ ○

9. … wurde ich von jemandem aus meiner Familie so stark geschlagen, dass ich ins Krankenhaus musste. ○ ○ ○ ○ ○

10. … gab es nichts, was ich an meiner Familie ändern wollte. ○ ○ ○ ○ ○

11. … schlugen mich Personen aus meiner Familie so stark, dass ich blaue Flecke oder Schrammen davontrug. ○ ○ ○ ○ ○

12. … wurde ich mit einem Gürtel, einem Stock, einem Riemen oder mit einem harten Gegenstand bestraft. ○ ○ ○ ○ ○

13. … gaben meine Familienangehörigen aufeinander Acht. ○ ○ ○ ○ ○

14. … sagten Personen aus meiner Familie verletzende oder beleidigende Dinge zu mir. ○ ○ ○ ○ ○

15. Ich glaube, ich bin körperlich misshandelt worden, als ich aufwuchs. ○ ○ ○ ○ ○

16. … hatte ich eine perfekte Kindheit. ○ ○ ○ ○ ○

17. … wurde ich so stark geschlagen oder verprügelt, dass es jemandem (z.B. Lehrer, Nachbar oder Arzt) auffiel. ○ ○ ○ ○ ○

	nie				sehr oft
	0	1	2	3	4

In meiner Kindheit …

18. … hatte ich das Gefühl, es hasste mich jemand in der Familie. ○ ○ ○ ○ ○

19. … fühlten sich meine Familienangehörigen einander nah. ○ ○ ○ ○ ○

20. … versuchte jemand, mich sexuell zu berühren oder mich dazu zu bringen, sie oder ihn sexuell zu berühren. ○ ○ ○ ○ ○

21. … drohte mir jemand, mir weh zu tun oder Lügen über mich zu erzählen, wenn ich keine sexuelle Handlung mit ihm oder ihr ausführen würde. ○ ○ ○ ○ ○

22. … hatte ich die beste Familie der Welt. ○ ○ ○ ○ ○

23. … versuchte jemand, mich dazu zu bringen, sexuelle Dinge zu tun oder bei sexuellen Dingen zuzusehen. ○ ○ ○ ○ ○

24. … belästigte mich jemand sexuell. ○ ○ ○ ○ ○

25. Ich glaube, ich bin emotional (gefühlsmäßig) missbraucht worden, als ich aufwuchs. ○ ○ ○ ○ ○

26. … gab es jemanden, der mich zum Arzt brachte, wenn es sein musste. ○ ○ ○ ○ ○

27. Ich glaube, ich bin während meiner Kindheit oder Jugendzeit sexuell missbraucht worden. ○ ○ ○ ○ ○

28. … war meine Familie mir eine Quelle der Unterstützung. ○ ○ ○ ○ ○

29. … waren meine Eltern (Stiefeltern) oder andere Personen aus meiner Familie unberechenbar. ○ ○ ○ ○ ○

30. … befürchte ich, dass meine Familie jederzeit auseinander brechen könnte. ○ ○ ○ ○ ○

31. … konnte ich mich meiner Familie nicht sicher fühlen. ○ ○ ○ ○ ○

Für den Versuchsleiter   (erst Fragebogen, dann Notizen)

Vorab: Auf dem Weg zum TSST-Raum nachfragen, was die VP nach dem Studium für einen Job ergreifen möchte. Alternativ: Was wäre der Traumberuf der VP.

Proband in den Raum führen.

„Sie werden jetzt ein Vorstellungsgespräch führen."

„Dies ist das Auswahl- Gremium. Diese Dame und dieser Herr sind zwei in der Verhaltensbeobachtung geschulte Psychologen, die gleich Ihr Verhalten analysieren werden. Hierzu dienen ihnen auch die Video- *(auf die Kamera deuten)* **und Tonaufnahmen** *(auf das Mikrophon zeigen)***. Auch Ihre Stimmfrequenz wird später mit Hilfe dieser Aufnahmen beurteilt.**

Stellen Sie sich nun bitte folgende Situation vor: Sie bewerben sich auf eine Stelle als XY *(hier Berufswunsch der Vp einsetzen)***, die Sie unbedingt haben möchten. Das Gremium soll anhand Ihres Vortrags beurteilen, ob und wie gut Sie für diese Stelle geeignet sind. Allerdings liegen dem Gremium Ihre Bewerbungsunterlagen wie Lebenslauf und Zeugnisse bereits vor, deshalb sollen Sie in Ihrem Vortrag nur Ihre persönlichen Eigenschaften vorstellen, die Sie für den Job gegenüber Ihren Mitbewerbern besonders qualifizieren. Wichtig ist, es handelt sich hierbei um eine freie Rede.**

Nach Ihrem Vortrag haben Sie noch eine weitere Aufgabe aus dem mathematischen Bereich zu lösen. Worum es sich dabei genau handelt, wird Ihnen jedoch das Gremium erst nach Ihrem Vortrag mitteilen.

Sie haben jetzt bis zu Ihrem Vortrag noch 5 Minuten Zeit. In diesen 5 Minuten füllen Sie bitte dort an dem Tisch *(auf den Vorbereitungstisch zeigen)* **zuerst einen kurzen Fragebogen aus, der Ihre Einstellung zu der gleich folgenden freien Redesituation erfragt. Ihre Antworten auf die dort gestellten Fragen werden nicht vom Gremium beurteilt. Nach dem Ausfüllen des Fragebogens haben Sie in der verbleibenden Zeit die Möglichkeit, sich Notizen zu Ihrem Vortrag zu machen. Die Notizen dürfen Sie allerdings während des Vortrags nicht verwenden. Haben Sie hierzu noch Fragen?**

Dann würde ich Sie bitten, einmal hier ans Mikrophon zu treten, damit ich es Ihrer Größe entsprechend einstellen kann *(Mikrophon jetzt auf die richtige Größe einstellen und dann schon mal langsam Richtung Tür gehen)***. Sie können sich jetzt an den Tisch setzen, die 5 Minuten Vorbereitungszeit beginnen, sobald ich den Raum verlassen habe."**

„Denken Sie daran, dass Sie den Job unbedingt haben möchten!" „Ich wünsche Ihnen viel Erfolg!" *(Versuchsleiter verlässt den Raum)*

TSST-Ablauf

Beginnt, nachdem der Versuchsleiter den Raum verlassen hat. Gremium lässt sich auf keine Gespräche ein. Wenn Vp früher aufsteht oder fragt wie viel Zeit noch ist, antworten, dass die Vorbereitungszeit noch nicht beendet ist.

Der Proband darf sich Notizen machen, diese aber NICHT beim Vortrag verwenden.

Teil 2: Bewerbungsgespräch (Minute 3 bis 8)

„Ihre Vorbereitungszeit ist jetzt zu Ende, bitte stellen Sie sich nun vor das Mikrofon."

Falls die Vp weit vom Mikro weg steht, Vp mitteilen, dass sie sich wegen der Ton-Aufzeichnung näher vor das Mikro stellen muss.

Wenn die Vp richtig steht, schaltet der gleichgeschlechtliche Gremiumspart die Kamera an. Bitte überprüfen, dass der Bildschirm an ist und Vp gut darauf sichtbar ist.

Sobald das Gremiumsmitglied wieder sitzt, teilt der andere mit:

„Bitte beginnen Sie jetzt mit Ihrem Vortrag"

Zunächst die Probanden solange wie möglich frei *über ihre Eigenschaften* sprechen lassen.

Wichtig: Bitte darauf achten, dass der Proband über seine Persönlichkeit spricht. Es besteht die Tendenz, fachliche Qualifikationen oder ähnliches darzubieten. In diesem Fall immer eingreifen:

„Ihr Lebenslauf/ Ihre Zeugnisse liegen uns bereits vor, bitte berichten Sie weiter über Ihre persönlichen Eigenschaften." Falls die Vp nichts mehr sagt, bzw. sagt, dass Sie nichts mehr zu Ihren Eigenschaften sagen kann, schweigen (gut sind 20 Sekunden), dann Hinweis

„Sie haben noch Zeit, bitte fahren Sie mit Ihren Eigenschaften fort". Bei einem erneuten Stocken des Vortrags wieder schweigen, danach erneuter Hinweis, dass die Zeit noch nicht vorbei sind (eventuell Aufforderung **„Kommen Sie noch auf Ihre negativen Eigenschaften zu sprechen"** / **"Kommen Sie noch mal auf Ihre negativen Eigenschaften zurück."**). Generell gilt:

Konkrete Fragen (siehe Liste) von Seiten des Gremiums so lange wie möglich vermeiden, da dies für die Vp eher eine Erleichterung darstellt! Auch auf Fragen von der Vp möglichst nicht eingehen, sondern auf die Aufgabe hinweisen und dass alles weitere nach dem Versuch geklärt wird.

- *„Warum halten gerade Sie sich für besonders geeignet für diese Aufgabe?"*
- *„Warum halten Sie sich selber für geeigneter als andere Bewerber?"*
- *„Sie wiesen gerade darauf hin, dass Sie besonders gut ... können, welche besonderen Eigenschaften zeichnen sie sonst noch aus?"*

- *„Sie haben gerade ihre besonderen Qualitäten in bezug auf ... aufgezeigt, welche typischen Eigenschaften zeichnen sie darüber hinaus aus?"*
- *„Vervollständigen Sie bitte den Satz 'ich bin der/die beste in ... '"*
- *„Sie sprachen gerade von ... , was halten Sie denn dann von ... ?"*
- *„Was schätzen Ihre Familie und Ihre Freunde besonders an Ihnen?"*
- *„Welche Führungsqualitäten besitzen Sie?"*

Teil 3: Rechenaufgabe (Minute 8 bis 13)

„Danke, das genügt. Wir haben nun noch eine weitere Aufgabe für Sie. Sie sollen nun von 2043 in 17- er Schritten so schnell und präzise wie möglich rückwärts zählen. Wenn Sie einen Fehler machen, werde ich Sie darauf hinweisen, dann beginnen Sie bitte wieder bei 2043. Haben Sie noch Fragen? Dann beginnen Sie bitte jetzt."

(Stoppuhr starten, ab hier 5 Minuten). Es werden während des Zählens keine Hilfen gegeben oder Fragen beantwortet. Bei einem Fehler lautet der Hinweis:

„Fehler- 2043 bitte" oder **„Fehler, bitte noch einmal von vorne"**

2043	2026	2009	1992	1975	1958	1941	1924	1907	1890	1873	1856	1839
1822	1805	1788	1771	1754	1737	1720	1703	1686	1669	1652	1635	1618
1601	1584	1567	1550	1533	1516	1499	1482	1465	1448	1431	1414	1397
1380	1363	1346	1329	1312	1295	1278	1261	1244	1227	1210	1193	1176
1159	1142	1125	1108	1091	1074	1057	1040	1023	1006	989	972	955
938	921	904	887	870	853	836	819	802	785	768	751	734
717	700	683	666	649	632	615	598	581	564	547	530	513
496	479	462	445	428	411	394	377	360	343	326	309	292
275	258	241	224	207	190	173	156	139	122	105	88	71
54	37	20	3									

Wenn die Probanden an die Decke o.ä. schauen:

„Bitte sprechen Sie näher ins Mikrofon!"

Wenn die Probanden keine Fehler machen:

„Bitte bemühen Sie Sich etwas schneller zu rechnen"

Darauf achten, dass die Probanden immer die komplette Zahl nennen (also nicht 2043 – 26 - 9 – 992 usw.)

Nach Ablauf der 5 Minuten:

„Vielen Dank, ich denke wir haben einen Eindruck gewonnen. Sie können nun den Raum verlassen. Der Versuchsleiter wartet vor dem Raum auf Sie."

Datum				Zeitplan		
Teilnehmer	♀ / ♂			**Vorbereitung**	:	**h**
Interviewer 1				**Rede**	:	**h**
Interviewer 2				**Rechnen**	:	**h**

TECHNISCHE UNIVERSITÄT DRESDEN

Fakultät Psychologie
Institut für Allgemeine Psychologie,
Biopsychologie und Methoden der
Psychologie

Professur Biopsychologie
Prof. Clemens Kirschbaum

	Zeitplan
Raus : **h**	

Gesprächsprotokoll – Teil 1: Freier Vortrag

Bitte ankreuzen:	☐	**AbX**	☐	**AvX**	☐	**BmQ**	☐	**FsQ**	☐

Sichtbare Symptome

Vortrags-qualitäten

Team- & Leadership

Gesprächsprotokoll – Teil 2: Rechnen

Bitte hier ausdrücklich die mathematischen Fähigkeiten des Bewerbers bewerten!

Bitte	☐	**math**	☐	**sub**	☐	**pQ**	☐	**hV**	☐
ankreuzen:	☐	**Fdj**	☐	**greX**	☐	**fun**	☐	**pert**	☐

Unterschrift

Gremium

Mini-DIPS Protokoll

Probleme/Schwierigkeiten allgemein

_____
_____

➡ *[PROBLEMBEREICH] Angst*
Paniksyndrom (PS):

_____
_____

Agoraphobie (AG):

_____
_____

Sozialphobie (SP):

_____
_____

Spezifische Phobien (SSP):

_____
_____

Generalisierte Angststörung (GAS):

_____
_____

Posttraumatische Belastungsstörung (PB):

_____
_____

➡ *[PROBLEMBEREICH] Zwang (ZS)*

_____
_____

➡ *[PROBLEMBEREICH] Affektive Störungen*
Schweres Depressives Syndrom (SDS)

_____
_____

Dysthyme Störungen (DS)

_____
_____

Manische Episode (ME)

_____
_____

➡ *[PROBLEMBEREICH] Somatoforme Störungen*
Hypochondrie (HYP)

_____
_____

Somatisierungssyndrom (SOM)

_____
_____

Schmerzsyndrom (SCH)

_____
_____

Konversionssyndrom (KS)

_____
_____

➡ *[PROBLEMBEREICH] Essstörungen*
Anorexia Nervosa

_____
_____

Bulimia Nervosa

_____
_____

➡ *[PROBLEMBEREICH] Alkohol, Medikamente und Drogen*

_____
_____
_____

➡ *Psychosen-Screening*

_____
_____

ABSCHLIESSENDE FRAGEN

_____
_____
_____

_

Empfangsbestätigung

Hiermit bestätige ich,

Name:_____

Vorname:_____

dass ich für die Teilnahme an einem psychologischen Experiment in der Abteilung Biopsychologie der Fachrichtung Psychologie der TU Dresden eine Aufwandsentschädigung von EUR 50,- erhalten habe.

Dresden, den _____

_____
Unterschrift Teilnehmer/in

_____
Unterschrift Versuchsleiter

Erklärung zum Stress-Test TSST

(Trierer Sozial Stress Test)

Wir möchten Sie hiermit darüber aufklären, dass eine wesentliche Voraussetzung für die Durchführung der Stress-Situation, die Sie heute erlebt haben, die **Neuheit** der Situation ist. Der wissenschaftliche Erfolg weiterer Untersuchungen mit diesem Stresstest hängt im Wesentlichen davon ab, dass die Situation auch für zukünftige Versuchsteilnehmer neu und unbekannt ist.

Wir würden Sie daher gerne bitten, potentiellen zukünftigen Teilnehmern – also gleichaltrigen Freunden, Kommilitonen, etc. – die Situation nicht detailliert zu beschreiben. Es ist unproblematisch, zukünftigen Teilnehmern zu erzählen, *dass* die Situation stressig wird, und *dass* eine Bewerbungssituation simuliert wird.

Problematisch wäre jedoch, wenn zukünftige Teilnehmer erfahren würden,

- o dass das Gremium **nicht** in Verhaltensbeobachtung geschult ist und sich auch **keine** personenbezogenen Notizen macht;
- o dass die Leistung im Stresstest **nicht** bewertet wird;
- o dass eine schwierige Rechenaufgabe zu lösen ist;
- o dass das Gremium **nicht** auf die Person des Vortragenden eingeht, bzw. kein Feedback gibt;
- o dass der Vortragende über seine eigene Persönlichkeit sprechen muss;

Ich erkläre hiermit, dass ich im Interesse des wissenschaftlichen Erkenntnisgewinns oben genannte Informationen über den Stress-Test „TSST" nicht an Freunde und Bekannte weitergebe.

Dresden, den _____

_____   _____
Unterschrift Teilnehmer   Unterschrift Versuchsleiter

Abriss

Hintergrund Im Jahr 2003 veröffentlichte Caspi et al. eine Studie die einen signifikanten Gen-Umwelt (G × U) Interaktionseffekt zwischen dem kurzen Allel des Serotonin Längenpolymorphismus (5-HTTLPR) und frühen traumatischen Erlebnissen (FTE) auf depressive Symptomatologie nachwies. Seither haben widersprüchliche Ergebnisse in Untersuchungen und Meta-analysen eine lebendige wissenschaftliche Debatte über die Existenz einer solchen G × U Interaktion befeuert. Ein Ansatz um die Komplexität der interagierenden Faktoren zu reduzieren ist die Verwendung von Endophänotypen. Ein solcher ist die Stress Reaktivität der Hypothalamus-Hypophyse-Nebennieren-Achse (HHN-Achse). Die HHN-Achse wird durch Gene und die Umwelt beeinflusst. Des weiteren weisen diverse psychiatrische Störungen eine Fehlregulation dieses Systems auf. Obwohl erste Studien dieses Ansatzes signifikante Effekte berichteten, wurde die angestrebte Erhöhung der erklärbaren Varianz nicht erzielt. Aus diesem Grund befürworten führende Forscher ein noch tieferes Eintauchen in die Schichten biologischer Information – epigenetische Profile können die Gentranskription direkt verändern und werden dynamisch durch Umweltsignale beeinflusst. Das Ziel der vorliegenden Studie ist es daher, den Einfluss von FTE und 5-HTTLPR auf das Niveau der Methylierung in der Promoterbereich assoziierten Region des Serotonintransporter Genes (*SLC6A4*) zu untersuchen. Zusätzlich, soll die Rolle von Methylierung im Kontext des 5-HTTLPR bei der HHN-Achsen Reaktivität während psychosozialem Stress erforscht werden.

Methode Junge (18 − 30 J) kaukasische Erwachsene (N = 186, 96 weiblich) wurden für den 5-HTTLPR und 5-HTTLPR/rs25331 mini Haplotyp genotypisiert. Um die Durchschnitts Methylierung der *SLC6A4* Promoterbereich assoziierten Region zu bestimmen, wurde DNA aus Vollblut entnommen, mit Bisulfiten behandelt und die DNA Abfolge mit Pyrosequenzierung bestimmt. Weiter wurde die Geschichte von FTE (Alter < 13) mit Hilfe des Childhood Trauma Questionnaire (CTQ) erfasst. Die Reaktion der HHN-Achse auf den Trier Sozial Stress Test (TSST) wurde durch Speichel Kortisolproben gemessen.

Resultate Es konnte ein signifikanter Gen − Epigenetik Interaktionseffekt zwischen dem 5-HTTLPR und der Durchschnitts Methylierung von 83 CpG-Orten in der von Philibert et al. (2008) definierten 799 BP langen CpG Insel festgestellt werden. Bezogen auf das S Allele zeigte die Gruppe mit ‚niedriger' Methylierung eine Dosis abhängige Steigerung der Kortisolsekretion, welche 7-9% der beobachteten Varianz

erklärte. Im Kontrast wies die ‚hohe' Methylierungs Gruppe keine Genotyp spezifischen Unterschiede bezüglich der HHN-Achsen Reaktivität auf. Vorhergehende Berichte über einen Zusammenhang zwischen früher Traumatisierung und Methylierungs Profil in der Promoterbereich assoziierten Region des *SLC6A4* konnten nicht bestätigt werden.

Schlussfolgerung Diese Ergebnisse unterstreichen die Relevanz von epigenetischen Daten in psychiatrischer Genetik und der Stressforschung. Die Einbeziehung von Methylierungsdaten offenbarte einen anderweitig verstecken Effekt und hat daher das Potential, unsere aktuellen Modelle zu verbessern. Diese Ergebnisse bleiben vorläufig bis weitere Replikationen publiziert werden.

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Praktika & Praxis

2015   Seit Jun   **Autisten Zentrum Arche Noah – Wien**
Betreuung von autistischen Menschen mit komorbiden psychiatrischen Erkrankungen (Schizophrenie, Bipolar, ADHS, Zwangsststörungen)

2013   Jun – Sep   **Wiss. Mitarbeit Biopsychologie TU-Dresden**
- Gen-Umwelt Interaktion im Kontext der Stresshormonregulation: die Bedeutung epigenetischer Prozesse
- Administration (Zeitplanung, Probanden Akquirierung & Korrespondenz, Ablauf)
- Diagnostik (DIPS) / Interviews

2012   Aug – Sep   **Institut klinische Psychologie & Psychotherapie TU-Dresden**
- Prof. Dr. Jürgen Hoyer – Ambulatorium Angststörungen (Therapiesitzungen, Supervision)
- fMRT Studien (Durchführung, Einweisung)
- Evaluationsforschung: Mindfulness Based Cognitive Therapy

2012   Jun – Aug   **Institut Biopsychologie TU – Dresden**
- Kortisol Haaranalysen
- Diagnostik & Stresstest Bundeswehr Soldaten
- Mitarbeit in der Vorbereitung zu Studie Epigenetik in der hormonellen Stress Forschung

2010-2011   Okt - Okt   **Anamnesegruppe Med. Universität Wien**
Interdisziplinäre Patienten Anamnese im AKH - Abteilung Onkologie & HIV Station (Gesprächsführung, Gruppenreflexion)

Lebenslauf

Studium

2015	Okt	Abschluss der Diplomarbeit „Genetic, epigenetic and environmental modulators of the human stress response"
2006		Beginn des Diplomstudiums der Psychologie an der Universität Wien

Fähigkeiten

Gesprächsführung
Sehr gute Englischkenntnisse   Sehr gute Computerkenntnisse

Interessen

Psychotherapie   Neurologie & Genetik
Meditation & Entspannungsverfahren   Philosophie & Religion
Physiologie & Sport