Hypothalamic-pituitary-adrenal (HPA) axis-related genetic variants influence the stress response

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III. List of Abbreviations

ACTH	Adrenocorticotropic hormone
AL	Allostatic load
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AUCi	area under the curve with respect to increase
AUCg	area under the curve with respect to ground
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
cAMP	Cyclic-adenosine monophosphate
CAR	Cortisol awaking response
cDNA	Complementary DNA
CGI	CpG islands
CpG	C—phosphate—G
CPT	Cold pressor test
CRE	cAMP response element
CREB	cAMP response-element binding
CRH	Corticotropin-releasing hormone
CRHBP	CRH binding protein
CORT	Cortisol
DBD	DNA-binding domain
DBP	Diastolic blood pressure
DST	Dexamethasone Suppression Test
EDTA	Dinatriummethylendiaminteraacetat
EM	Expectation-maximisation
EMEM	Eagle’s Minimal Essential Medium
emQTLs	expression methylation Quantitative Trait Loci
eQTLs	expression Quantitative Trait Loci
eQTMs	expression Quantitative Trait Methylation
FBS	Fetal Bovine Serum
fMRI	Functional magnetic resonance imaging
Fwd	Forward or sense primer
GAS	General Adaptation Syndrome
GCs	glucocorticoids
GFP	Green flurescence protein
GLM	General linear model

GR	Glucocorticoid receptor
HDL	High-density lipoprotein
HPA	Hypothalamic-pituitary-adrenal
HR	Heart rate
HSP	Heat shock proteins
HWE	Hardy-Weinberg equilibrium
LBD	Ligand-binding domain
LD	Linkage disequilibrium
mQTLs	methylation Quantitative Trait Loci
NR3C1	Nuclear receptor subfamily 3, group 3, member 1
NR3C2	Nuclear receptor subfamily 3, group 3, member 2
NTD	N-terminal domain
PBMC	Peripheral blood mononuclear cells
PSNS	Parasympathetic nervous system
PTSD	Posttraumatic stress disorder
PVN	Paraventricular nucleus
QTL	Quantitative trait locus
Rev	reverse or antisense primer
RLU	Relative light units
rmQTLs	stress-response methylation Quantitative Trait Loci
rQTLs	stress-response Quantitative Trait Loci
rQTMs	stress-response Quantitative Trait Methylation
SAM	Sympathetic-adrenal-medullary
SBP	Systolic blood pressure
SeCPT	Socially-evaluated cold pressor test
SEM	SNP-expression-methylation model
SMR	SNP-methylation stress response
SNP	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
TA	Transcription activity
TC	Total cholesterol
TF	Transcription factors
TICS	Trier Inventory for Chronic Stress
Tm	annealing temperature in PCR
TSST	Trier Social Stress Test
UTR	Untranslated region
VIF	Variance inflation factors

VNTR	Variable number of tandem repeats
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine transporter (serotonin transporter)
5-HTTLPR	5-hydroxytryptamine-transporter-linked polymorphic region

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Outline

The physiological stress system includes the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary system (SAM). Parameters representing these systems such as cortisol, blood pressure or heart rate define the physiological reaction in response to a stressor. The main objective of the studies described in this thesis was to understand the role of the HPA-related genetic factors in these two systems. Genetic factors represent one of the components causing individual variations in physiological stress parameters. Five genes involved in the functioning of the HPA axis regarding stress responses are examined in this thesis. They are: corticotropin-releasing hormone (*CRH*), the glucocorticoid receptor (*GR*), the mineralocorticoid receptor (*MR*), the 5-hydroxytryptamine-transporter-linked polymorphic region (5-*HTTLPR*) in the serotonin transporter (5-*HTT*) and the brain-derived neurotrophic factor (*BDNF*) gene. Two hundred thirty-two healthy participants were genotyped. The influence of genetic factors on physiological parameters, such as post-awakening cortisol and blood pressure was assessed, as well as the influence of genetic factors on stress reactivity in response to a socially evaluated cold pressor test (SeCPT). Three studies tested the HPA-related genes each on three different levels. The first study examined the influences of genotypes and haplotypes of these five genes on physiological as well as psychological stress indicators (**Chapter 2**). The second study examined the effects of *GR* variants (genotypes and haplotypes) and promoter methylation level on both the SAM system and the HPA axis stress reactivity (**Chapter 3**). The third study comprised the characterization of *CRH* promoter haplotypes in an *in-vitro* study and the association of the *CRH* promoter with stress indicators *in vivo* (**Chapter 4**).

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Chapter 1 General Introduction

1.1. The basics of the stress response: Historical context and introduction

One-hundred years ago, ***stress*** (Linkowski et al., 1993) was a term used by engineers referring to forces that put a strain on a structure (Keil, 2004). It did not possess its contemporary meaning yet. Only in the 1920s, it started to become an occasionally used term when biologists or psychologists referred to mental or environmental causes of certain illnesses. Today, stress has become an essential topic in diverse areas of the behavioral, social, and life sciences. Table1.1 delineates the landmarks in the development of stress research.

Table 1.1: Milestones in stress research.

Year	Person	Milestones	References
1929	Walter Cannon	He coined the term fight-or-flight response and expands on Claude Bernard's concept of homeostasis.	(Cannon, 1929; Cannon, 1932)
1956	Hans Selye	He expanded on Cannon's work by investigating the hypothalamic-pituitary-adrenal (HPA) axis and coined the concept of a General Adaptation Syndrome (GAS). His invaluable contributions to the field were pioneering the exploration of the relationship between the physiology of glucocorticoids (GCs) and stress.	(Selye, 1956)
1975	John Mason	He discovered that the GAS response could be modified based on situational and emotional factors, and that psychological interpretation of a stressor is necessary for the subsequent endocrine response to occur.	(Mason, 1975)
1984	Munck et al.	They proposed a novel way to think about the role of GCs in the stress response that countered Selye's general viewpoint that GCs direct the stress response. They hypothesised that GCs work to suppress, rather than enhance, the standard defence mechanisms. In other words, GCs confer protection from the stress response.	(Munck, Guyre, & Holbrook, 1984).
1985	Richard Lazarus	He demonstrated that individual differences in various cognitive and motivational variables, such as appraisal and coping, can arbitrate the relationship between a stressor and the stress reaction. Thus, an evaluation of stressors determines their level of threat and their subsequent ability to elicit a stress response.	(Lazarus, 1985)

1988	Sterling and Eyer	They introduced a novel conception called allostasis to the stress field, to be distinguished from homeostasis.	(Sterling & Eyer, 1988)
1992	Chrousos and Gold	They integrated the concept of individual differences based on genetic factors as an essential consideration for measuring the stress response.	(Chrousos & Gold, 1992).
1993	Kirschbaum et al.	A standardised stressor, the Trier Social Stress Test (TSST) was developed to challenge the HPA axis under controlled conditions.	(Kirschbaum, Pirke, & Hellhammer, 1993).
1997	Linkowski et al.	A reliable biological marker, the salivary cortisol awaking response (CAR) was characterised by an increase in cortisol during the first 30 minutes after the individual awakens, followed by a decrease in secretion. It is used for the assessment of adrenocortical activity.	(Linkowski et al., 1993; Pruessner et al., 1997; Späth-Schwalbe et al.,1991).
1998	Bruce McEwen	He expanded on the concept of allostasis (McEwen, 1998). Allostasis refers to the ability of the body to achieve and maintain stability through change and represents an adaptive coping mechanism in which various stress response processes are engaged during stress.	(McEwen, 2000)
2000	Sapolsky et al.	They extended Munck's model of GC by distinguishing explicitly between rapid and relatively slow, the circadian action of GCs, thus, including a more comprehensive set of preparative, simulative, permissive, and suppressive functions of GCs.	(Sapolsky, Romero, & Munck, 2000)
2005	Karst et al.	They reported non-genomic effects mediated via classical mineralocorticoid receptor.	(Karst et al., 2005).
2008	Schwabe et al.	They developed the socially-evaluated cold pressor test (SeCPT), a convenient and economical tool for inducing stress in the lab.	(Schwabe et al., 2008; Schwabe & Schächinger, 2018).
2009	Cirimele et al.	Methods for determining human hair cortisol concentrations were assumed to make available a retrospective reflection of integrated cortisol secretion over periods of several months.	(Cirimele et al., 2000; Clemens et al., 2009; Koren et al., 2002; Sauvé et al., 2007).

In the history of stress research, four main steps of development can be distinguished.

First, the early understanding of general stress (such as GAS) identified three essential elements: input (stressor), processing of stressful information, and output (stress response) (Selye, 1956).

Second, the development of a dynamic stress concept seeking to achieve balance replaced earlier views of stress as static. The stress process involves several biological and psychological systems, such as the hypothalamic-pituitary-adrenal (HPA) axis or the sympathetic-adrenal-medullary (SAM) autonomous nervous system. Moreover, the original homeostasis concept has been refined into an allostasis concept (Sterling & Eyer, 1988).

Third, individual differences were taken into account, in particular, personal coping processes that activate compensatory psychological and biological factors. Those individual variables rooted in environmental factors, (e.g. upbringing, socioeconomic status), as well as psychological factors (e.g. personality, temperament, cognitive process) (Mason, 1975; Lazarus, 1985). Furthermore, biological differences are derived from genetic factors, such as genotype, gene expression level, and epigenetic mechanisms (Chrousos & Gold, 1992).

Fourth, stress research aims at identifying the potential role of stress in the development and maintenance of health problems. Technological advances in measurement together with methods of inducing stress in the laboratory allow us to find some stress-level markers and thereby identify how stress is associated with diseases and health.

Following these four steps, in **Chapter 1**, the stressor (1.1.1) and stress responses (1.1.2) will be introduced. The major physiological stress systems, the HPA axis and the SAM system, will be briefly introduced and the relationship between both will be elaborated in the subchapter. The parameters of the HPA axis and the SAM system in lab research will be summarised. Finally, subjective psychological stress-related questionnaires will be shortly introduced.

1.1.1 Stressor

Within the stress concept, stressors are the input element that trigger processing of stressful information and ultimately result in a stress response (Fink, 2000). There are several groups of stressors, not only regarding their intensity and duration (acute versus chronic) but also their character. Regarding character, a stressor may be a chemical or biological agent, an environmental condition, an external stimulus or an event (Fink, 2000). Stressors may be divided into physical stressors (e.g., cold, heat, intense radiation, vibration, and many others), chemical stressors (e.g., ether, drugs, tobacco, alcohol and all poisons), social stressors (e.g., societal or family demands), and psychological stressors that affect emotional processes and may result in behavioral change (e.g. anxiety, fear, or frustration) (Fink, 2000). In general, these psychological stressors have been classified into four categories 1) crises/catastrophes (e.g., natural disasters and wars), 2) major life events (e.g., marriage, death of a loved one, birth of a child), 3) daily hassles/microstressors (e.g., traffic jams, meeting deadlines at work). 4) ambient stressors (e.g., pollution or noise). Importantly, psychological stressors vary among individuals. Not everybody perceives the same event as equally stressful. Therefore, individual differences must be regarded as a prime factor in stress research.

1.1.2 Stress response

Manifold adaptive responses include physiological and behavioral processes that are affected by primary stress effector systems. One of the primary stress effector systems is the sympathetic-adrenal-medullary (SAM) system that is responsible for the release of norepinephrine and epinephrine. The second one is the hypothalamic-pituitary-adrenal (HPA) axis secreting corticotropin-releasing hormone (CRH), adrenoadrenocorticotrophic hormone (ACTH) and various glucocorticoids (GCs) (Eysenck, 2004). Another primary stress effector system is the parasympathetic nervous system releasing acetylcholine (Kronenberg, Polonsky, Larsen, & Melmed, 2007). The fourth system is the renin-angiotensin system, in which renin is released from juxtaglomerular cells in the kidney (Kronenberg, Polonsky, Larsen, & Melmed, 2007). In this

thesis, the focus will be on the HPA axis and the SAM system. Parameters representing these two systems (e.g. cortisol, blood pressure, heart rate) define physiological reactions in stress responses.

1.1.2.1 Hypothalamic-Pituitary-Adrenal (HPA) axis

The HPA axis comprises direct effects as well as feedback interactions of three main components: hypothalamus, pituitary gland, and adrenal gland. The hypothalamus produces CRH when stressors affect the body. CRH stimulates the pituitary gland that releases ACTH. ACTH then activates the adrenal gland cortex to produce various GCs. Activation of the HPA raises plasma GCs exerting various metabolic effects (Kronenberg, Polonsky, Larsen, & Melmed, 2007). The mobilization of energy serves allostasis and survival of the organism. These metabolic effects comprise proteolysis, gluconeogenesis, inhibition of lipogenesis and glycogenolysis, lipolysis. Additional regulatory effects are, for example, anti-allergic or anti-inflammatory reactions. GCs exert effects in the adrenal gland (e.g. inhibition of GC synthesis, and the anterior pituitary inhibition of corticotrophs), and the hypothalamus (e.g. inhibition of CRH synthesis and secretion) (Kronenberg, Polonsky, Larsen, & Melmed, 2007). Effects can be preparative, stimulative, permissive, or suppressive (Sapolsky et al., 2000).

1.1.2.2 Sympathetic-Adrenal-Medullary (SAM) system

The SAM system involves the sympathetic branch of the autonomic nervous system (ANS). The SAM system connects the sympathetic nervous system (SNS) and the adrenal medulla (Eysenck, 2004). In response to sensory input, the SNS sends a signal to preganglionic nerve fibres, which activate the adrenal medulla through acetylcholine. When the adrenal medulla is stimulated, it secretes the hormones epinephrine and norepinephrine (also called adrenaline and noradrenaline respectively), which was known collectively as catecholamines, directly into the blood (Eysenck, 2004). These hormones arouse the SNS and simultaneously reduce the activity of the parasympathetic nervous system (PSNS). Thus, the SAM axis prepares us for a fight-or-flight response by heightened alertness, an increase in energy, more blood in the muscles,

reduced digestive activity, quicker heart rate, accelerated respiration, and stronger release of clotting factors into the bloodstream. Additionally, the output of the heart rises and increases blood pressure (Eysenck, 2004).

1.1.2.3 Relationships between the HPA axis and the SAM system

The HPA axis and the SAM system are two important physiological stress-responsive systems. There is general agreement among researchers that the SAM system is considered as an immediate response, whereas the HPA axis represents a longer-term response (Eysenck, 2004). However, the fact that they have been described as separate systems should not obscure the true reality. The HPA axis and the SAM system do not operate in complete independence from each other. As Evans (1998, page 60) points out, "At the level of the central nervous system, the crucially important SAM and HPA systems can be considered as one complex: they are as it were the lower limbs of one body." Close interaction between the HPA axis and the SAM system is suggested, for example, by the heightened vulnerability to develop addictions because of high sensitivity to cortisol effects mediated via mesencephalic dopaminergic neuron activity (Piazza & Le Moal, 1996). Moreover, circulating catecholamines affect CRH and AVP secretion in the hypothalamus as well as ACTH secretion in the pituitary (Plotsky, Cunningham, & Widmaier, 1989). Yet, both systems closely interact with each other, while they also differ in responses and sensitisation. A central aim of this thesis was to elucidate whether these complex systems and their responses to varying demands can be linked by genetic factors.

1.1.3 Psychophysiological research methods and tools in the laboratory

More and more studies show a link between psychological stress and morbidity. However, the mechanisms by which biological system, personal life experience and environmental demands implicated in the stress-related diseases interact remain unclear. One approach to address this issue is using psychophysiological research methods for investigating the effects of various stressors on specific biological markers. A great number of studies followed this approach, examining a wide array of outcomes, such as heart rate, blood pressure or salivary cortisol. The

following sections will describe the methods of testing in the stress psychophysiology laboratory including parameters of the HPA axis, parameters of the SAM axis, and psychological stress-related questionnaires.

1.1.3.1 Measures of hypothalamic-pituitary-adrenal activity

Three levels of HPA axis regulation can be differentiated: circadian rhythmicity, negative feedback and stress-induced activation. Several methods are developed to test these different aspects of the HPA axis in humans. Diurnal cortisol secretion and morning awakening cortisol levels can be tested at the basal non-stressful situation. Moreover, the reactivity of the HPA axis can be assessed either pharmacologically, by inducing stress in the laboratory, or through stressful events in real life outside the laboratory. In addition, synthetic glucocorticoids inhibiting the HPA axis can be used to test the negative feedback of this system. In table 1.2, these different assessments are summarized.

Table 1.2: Methods to evaluate the HPA in stress research

		Test	Method	Assessment	Advantages	Limitations
Basal HPA		Diurnal cortisol secretion	Saliva or serum sampling during the day	Circadian rhythm	The robust impression of day rhythm Mean cortisol level during the day	Low sensitivity for detecting psychopathology in psychiatric patients No evaluation of stress response Intensive for patients, serum sampling requires admission. (Kirschbaum & Hellhammer, 1989)
		Urinary free cortisol	Mean cortisol in urine 24h sampling	Total cortisol production (a marker for Cushings' syndrome)	Comparison of inter-individual differences Information on total cortisol production	No information on the stress response, no information on ultradian or circadian changes Increasingly unpopular due to low sensitivity as well as specificity in detecting Cushings' syndrome, however still broadly applied for diagnostic purposes (Burke & Beardwell, 1973)
		The cortisol awakening response (CAR)	Five measures within the first hour after awakening (saliva or serum)	Area-under-the-curve measures are indicating basal activity as well as HPA reactivity.	Associated with mood disorders non-invasive, outpatients, home and non-stress situations	Highly dependent on the time of awakening Compliance Strict requirements for data analysis (Linkowski et al., 1993; Pruessner et al., 1997; Späth-Schwalbe et al., 1991).
		Scalp hair cortisol	Cortisol levels in scalp hair strands	Long-term cortisol (months to years)	Easy, non-invasive, information on long-term mean systemic cortisol levels. Not affected by daily fluctuations Possibility to assess cortisol in retrospect	New method: lack of standardization of the unit of measurement Hair washing frequency and hair treatment influence the results. (Cirimele et al., 2000; Kirschbaum et al., 2009; Koren et al., 2002; Sauvé et al., 2007, Wright et al., 2015, Stalder et al., 2017)
HPA Reactivity	**Pharmacological challenges**	The CRH stimulation test	Administration of synthetic CRH (intravenous or intramuscular)	Diagnostic tool for anterior pituitary insufficiency	High sensitivity and specificity	Narrow indication, no information about feedback functioning. Invasive (Holsboer et al., 1986)
		The ACTH stimulation test	Administration of synthetic ACTH (intravenous or intramuscular)	Diagnostic tool for primary adrenal insufficiency	High sensitivity and specificity	Narrow indication, no information about feedback functioning. Invasive (Gold et al., 1988)

HPA Reactivity	Induced by real-life stressors	Environmental stress measures (ESM)	Saliva sampling in daily life in combination with self-report of daily life experience	HPA axis responsivity to everyday events	No recall bias Associated with the reduced flexibility of stress response and higher overall cortisol levels in bipolar patients	Depending on compliance, time consuming and demanding for participants (Biondi & Picardi1999)
	Laboratory-induced stressors	The Cold pressor test (CPT)	Physiological stress test under laboratory conditions	Cortisol levels as well as other HPA-axis parameters before, during and after acute stress	A tool to measure cardiovascular reactivity in response to stress	Less capable to provoke the HPA axis (Hines & Brown 1932; Roy-Gagnon et al., 2008)
		The Trier Social Stress Test (TSST)	Psychosocial stress induction under laboratory conditions	Cortisol levels as well as other HPA-axis parameters before, during and after acute stress	Induced “real” moderate to severe stress Standardized Tools Responsiveness of endocrine and immunological parameters	Ethical Considerations in patient populations Time consuming, expensive (Kirschbaum, Pirke, & Hellhammer, 1993).
		The Socially-evaluated cold pressor test (SeCPT)	Psychosocial stress test under laboratory conditions	Cortisol levels as well as other HPA-axis parameters before, during and after acute stress	A very convenient and economic tool in stress research	Only minor effect on HPA axis compared to TSST (Schwabe et al. 2008; Schwabe & Schächinger, 2018).
	Feedback regulation of the HPA axis	The Dexamethasone Suppression Test (DST)	Application of Dex in the evening; assessment of morning cortisol the next day	Negative feedback of central regulation	Easy to perform 1-mg DST is valuable in diagnosing Cushing’s syndrome 0.25 mg DST is valuable in determining GR sensitivity in healthy individuals	Low sensitivity in psychiatric populations (<45%) (Carroll et al., 1968)
		The Dexamethasone-CRH (Dex-CRH) test	1.5 mg Dex at 11 pm followed by 100 µg CRH at 3 pm next day, blood sampling from 2 pm- 6 pm	Negative feedback of central regulation	High sensitivity in psychiatric inpatients (80-90%) Higher cortisol levels predictive for relapse depression	Not suited for outpatients due to frequent sampling; expensive in large cohorts (Heuser et al., 1994; Bardeleben & Holsboer, 1989)
		Metyrapone test	Blocking cortisol production by inhibition of 11-deoxycortisol into cortisol	Diagnostic tool for testing the HPA-axis functioning	Widely used in endocrinology to diagnose secondary adrenal insufficiency Compensatory increasing ACTH levels indicate proper functioning of HPA axis on pituitary level	Not suited for subtle changes in HPA axis regulation (Tarek et al., 1994)
		Insulin tolerance test	By inducing hypoglycemia the HPA axis is strongly activated	Diagnostic tool for testing the HPA axis functioning	Diagnosing adrenal insufficiency	Risk for lethal hypoglycemia; should always be performed in clinical settings with close monitoring of vital functions. (Greenwood et al., 1966; Plumpton & Besser, 1969)

1.1.3.2 Measures of the SAM system

Until now, there are several proven methods to investigate the SAM system during stress response *in vivo*. Blood pressure, vagal tone and heart rate can directly provide insight into the SAM system. Salivary alpha-amylase level is also known as a potential indicator of noradrenergic activity. In table 1.3 these different assessments are summarised.

Table 1.3: Methods to evaluate the SAM system in stress research

Test	Method	Assessment	Advantages	Disadvantages
Blood pressure (Ogedegbe & Pickering, 2010)	A measure of the force that blood exerts on the walls of blood vessels	Assessment of sympathetic parameter by systolic blood pressure (SBP) and diastolic blood pressure (DBP).	One of the principal vital signs Easily measured using simple vital signs monitor Easy to perform	Average values for any given population will miss some information, due to a considerable variation in inter- and intra-individual difference. Various factors influence a person's average BP and variations.
Vagal Tone (Bentson, et al., 1993)	A measure of a break to decrease heart rate during both resting and reactive conditions.	Represents the parasympathetic impulse	More information on the parasympathetic system	Requires the use of more advanced electronic devices
Heart rate (Hall, 2015)	A measure of the speed of the heartbeat	Assessment of sympathetic and parasympathetic input parameter	Easy to perform	Values are generally considered average but depend greatly on individual physiology and fitness
Salivary alpha-amylase (Chatterton et al. 1996; Nater et al., 2005; Rohleder et al. 2004)	A measure of Alpha-amylase level in saliva	An indicator of noradrenergic activity	A potential biomarker for gauging the level for the activity of the sympathetic nervous system.	There are multiple confounders of this assessment; Compliance

1.1.3.3 Psychological stress-related questionnaires

In accordance with the definition of stress responses by Mason (1975) as a psychological concept, numerous questionnaires have been developed that cover a variety of psychological symptoms related to chronic stress. Examples of such questionnaires are the Daily Stress Inventory (Brantley et al., 1987), the Perceived Stress Scale (Cohen, Kamarck & Mermelstein, 1983), and the Trier Inventory for the Assessment of Chronic Stress (TICS) (Schulz & Schlotz, 1999). The present study used the TICS that consists of nine subscales (work overload, social overload, the pressure to succeed, work discontent, excessive demands from work, lack of social recognition, social tensions, social isolation and chronic stress worrying). Work overload, social overload, and pressure to succeed can be grouped into stress from higher demands. The scales work discontent, excessive demands from work, lack of social recognition, social tensions, and social isolation are all characterised by “deficits”. The third block contains only chronic worrying (Schulz & Schlotz, 1999).

1.2. Genetic variance in the HPA axis and association studies

People differ regarding their stress response systems. The genetic background modulates stress reactivity, as do early experience (Chrousos & Gold, 1992). The individual vulnerability can be defined at four levels: the psychological phenotype (e.g. personality); the functional phenotype (e.g. neuroendocrine responses); the genetic variations (e.g. polymorphisms in genes that contribute to HPA signaling); the epigenetic variations (e.g. DNA methylation levels in genes involved in the stress response pathway). Supplementing the psychophysiological approach with genetic analyses may allow a better distinction between the HPA and SAM effects and clarify how they interact with each other. In the following section, genetic variance in relation to the HPA axis will be introduced. Afterwards, five candidate genes will be detailed and accompanied by summaries of previous association studies.

1.2.1 Genetic variance

Biochemically all humans are estimated to be 99.6% (Tishkoff & Kidd, 2004) to 99.99% (Jorde & Wooding, 2004) similar to each other. However, multiple variants of genes, called alleles, exist in the human population. Genetic variations between populations occur mainly through DNA mutation, gene flow and genetic recombination (Hartl & Jones, 1998). Genetic variations take place on many scales, from single nucleotide changes to complete duplications of a chromosome. This thesis deals only with two genetic variations: Single nucleotide polymorphisms (SNPs) and a variable number of tandem repeats (VNTRs). Moreover, genotype, haplotype and epigenetic variation (DNA methylation) will be investigated in this thesis.

1.2.1.1 Single nucleotide polymorphisms (SNPs)

SNPs are the commonest type of genetic variation. They occur every 100 to 300 bases (Collins, Brooks, & Chakravarti, 1998) and are defined as a variation in a single nucleotide that occurs at a specific position in the genome between members of one species. The respective nucleotide can be inserted, substituted, or deleted. With about 3 billion nucleotides in the human genome, there are 10 to 30 million SNPs. SNPs may be located within non-coding or coding sequences of genes, as well as in the intergenic regions between genes. Synonymous (or neutral) SNPs do not affect the protein sequence by the degeneracy of the genetic code, whereas nonsynonymous SNPs alter amino-acid sequences of proteins. Nonsynonymous changes are further classified into nonsense and missense. Nonsense changes entail a premature stop codon. Missense changes result in a different amino acid. Only 3-5% of human SNPs are functional (see http://hapmap.ncbi.nlm.nih.gov/). Nonsynonymous SNPs also influence gene splicing, and so cause phenotypic differences. Moreover, in non-protein-coding regions, SNPs still may modulate gene expression through promoter activity, transcription factor binding, transcription efficiency, mRNA stability, or mRNA binding, as well as by effects on translation efficacy (Conrad, 2011; Kimchi-Sarfaty et al., 2007).

1.2.1.2 Variable number of tandem repeats (VNTRs)

VNTRs are variations of the length of a tandem repeat. A tandem repeat consists of the adjacent repetition of a short (ranging in length from 1 – 6 or more base pairs) nucleotide sequence. Many chromosomes possess tandem repeats. Their length varies between alleles of a gene and between individuals. Each variant acts as an inherited allele (Hartl & Jones, 1998). Therefore they can serve for personal and parental identification. They can be used in biological research, in forensics, and as DNA fingerprinting.

1.2.1.3 Haplotype

The term "haplotype" stems from "haploid" (denominating a cell with one set of chromosomes only) (Lewin, 2004). A haplotype is the group of the allele that a progeny inherited together from one parent. This group of alleles was inherited together because of genetic linkage, the phenomenon by which genes are close to each other on the same chromosome and often inherited together (Lewin, 2004). In addition, the term haplotype denominates also a cluster of SNPs on a single chromatid of a chromosome pair that is associated statistically. An organism's genotype may not define its haplotype uniquely. The unequivocal method of resolving phase ambiguity is sequencing, which is a currently time-consuming and expensive method. Therefore, often statistical methods are used for inferring haplotypes. Several models have been proposed, notably Clark's parsimony algorithm (Clark, 1990), the expectation-maximisation algorithm (EM) (Excoffier & Slatkin, 1995) and coalescence-based Bayesian haplotype inference (Niu et al., 2002). In this thesis, one algorithm based on coalescence-based Bayesian haplotype inference will be used (PHASE, version 2.1, http://c4c.uwc4c.com/express_license_technologies/phase) for inferring haplotypes from genotype data. In comparisons to using real and simulated data, this coalescence-based Bayesian algorithm outperformed numerous alternative methods (Stephens & Donnelly, 2003).

1.2.1.4 Epigenetics

The term “epigenetics” was first introduced by Conrad Hal Waddington (1940) to describe the study of causal analyses of development (Slack, 2002). Today, epigenetics refers to the study of heritable and reversible changes in organisms caused by modification of gene expression rather than alteration of the original DNA sequence (Bird, 2007). Several types of modifications have been reported as epigenetic markers, such as DNA methylation, histone modifications, and small RNAs. This thesis will focus on DNA methylation, which is a modification introducing a methyl group (the alkyl radical-CH3) into the fifth carbon position of a cytosine ring that is adjacent to guanines (CpG, "CpG" is only one phosphate separates shorthand for "C-phosphate-G", that is, cytosine and guanine) dinucleotides. The enzymatic machinery responsible for the maintenance of DNA methylation is established by the DNA methyltransferase 1 (DNMT1) family, and *de novo* methylation is carried out by the DNMT3 family. Mammalian DNA methylation levels are generally high, although CpG rich regions, called CpG islands (CGI), appear mostly unmethylated (Bird, 2002; Lister et al., 2009). Many cellular processes involve DNA methylation, including 1) gene expression 2) imprinting 3) X chromosome inactivation 4) silencing of retroviral and transposable DNA elements 5) chromatin organization (Sadikovic et al., 2008). Regulation of gene expression is vital for the body's tolerance for stress. In this context, DNA methylation is gaining interest because it may regulate the expression of genes that have a crucial role in stress responses. However, we still have a limited understanding of when, where, and how DNA methylation participates in gene expression. The relationships between DNA methylation and gene expression in a population context have been reported to be both positive and negative (Bell et al., 2011). Albeit DNA methylation is typically associated to gene silencing, recent studies suggest that it has distinct functions in gene regulation (Gutierrez-Arcelus et al., 2015). DNA methylation in gene bodies can be positively associated to gene transcription in a tissue-specific manner (Jones, 2012; Lister et al., 2009). DNA methylation can also regulate cell-context-specific alternative promoters

in gene bodies (Maunakea et al., 2010). It can also be a marker of tissue-specific regulatory elements (Ziller et al., 2013). Additionally, DNA methylation levels can be locally influenced by transcription factors (TFs) binding at enhancers (Stadler et al., 2011). Moreover, exons are more highly methylated than introns, and sharp transitions of methylation occur at exon-intron boundaries, suggesting a role for differential methylation in transcript splicing (Laurent et al., 2010)

1.2.2 Candidate genes of the HPA axis for stress regulation

To investigate the function of the HPA axis in response to a stressor, plausible genes that have been previously shown to be involved in the regulation of the HPA axis are selected to study in this thesis (Figure.1.1). They are: corticotropin-releasing hormone (CRH, gene symbol *CRH*; OMIM * 122560), the glucocorticoid receptor (GR, NR3C1; gene symbols *GR*, *NR3C1*; OMIM *138040), the mineralocorticoid receptor (MR, NR3C2; gene symbols *MR, NR3C2*; OMIM *600983), the 5-hydroxytryptamine-transporter-linked polymorphic region (5-HTTLPR) in the serotonin transporter gene (5-HTT, SLC6A4; gene symbols *5-HTT*, *SLC6A4*, OMIM *182138) and the brain-derived neurotrophic factor (BDNF, gene symbol *BDNF*; OMIM *113505). The CRH is the primary hypothalamic neurohormone. Glucocorticoid sensitivity represents a key index for individual differences in the HPA function (Désautés et al., 2002). Responses of the HPA axis exerted by cortisol are mediated by GR and MR. The GR serves as a transcription factor to facilitate metabolism following acute stress. The MR is involved in the appraisal of novel situations and the selection of appropriate coping responses (DeRijk, De Kloet, Zitman, & Van Leeuwen, 2011). Besides the glucocorticoid-driven modulation of the HPA axis, serotonergic projections directly facilitate or inhibit HPA axis activity. Activation of CRH neurons as the initial impetus of the HPA axis is regulated by the serotonergic pathway (Lowry, 2002). Therefore, the 5-HTT might play a role in CRH release from pituitary neurons and the subsequent cortisol release. Moreover, the basal, unstimulated and unstressed HPA axis is further affected via indirect hippocampal input to the hypophysiotrophicneurons regulating the hypothalamic CRH

expression. The BDNF contributes to the neuroplasticity of the hippocampus and the development of feedback mechanisms on the HPA axis response to stress (Shalev et al., 2009). Additionally, the functional characterization of three *CRH* promoter haplotypes and examination of the methylation level of *GR* promoter will be performed in this thesis. The detailed description of each gene can be found in the following section.

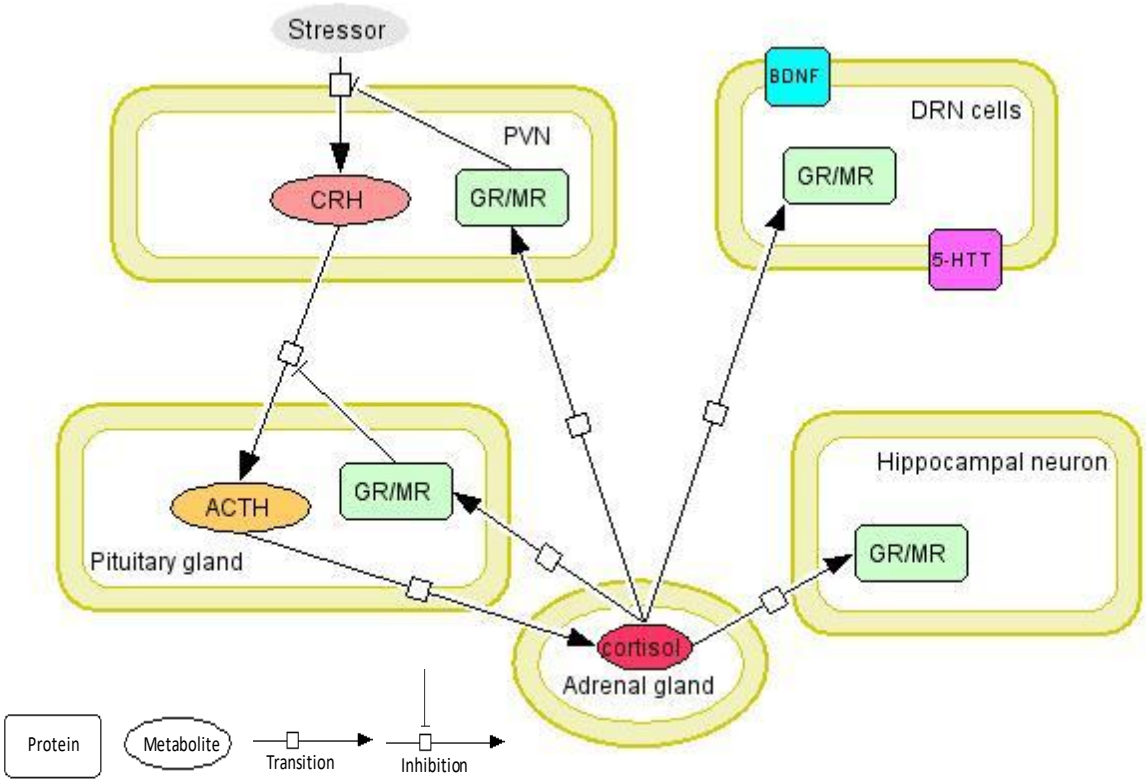

Figure 1.1 A figure illustrating the proteins encoded by candidate genes *CRH*, *GR*, *MR*, *BDNF* and 5-*HTT*, which are involved in the functioning of the HPA axis examined in this thesis. Possible genetic pathways of stress regulation in five different regions are depicted. This figure was generated using the computational program Cell Designer 4.4 (The Systems Biology Institute, Tokyo, Japan). DRN cells: dorsal Raphe nucleus. PVN: Paraventricular nucleus.

1.2.2.1 Corticotropin-releasing hormone (*CRH*)

CRH (also called corticotropin-releasing factor, *CRF*, or corticoliberin) is a major regulator of homeostasis and mediates the autonomic, behavioral and neuroendocrine responses to stress. The CRH release is the key event that leads to the actual activation of the HPA axis including the subsequent cortisol release. Cortisol binds to the GR and this complex exerts negative feedback at the level of CRH neurons, with direct or indirect binding to response elements at the

CRH promoter (Yao & Denver, 2007). This regulates *CRH* gene expression via complex actions (e.g.steroid actions and protein-protein interactions) (Aguilera, G. et al. 2007).

Genetic architecture

CRH belongs to the corticotropin-releasing factor family. In humans, it is encoded by the *CRH* gene, which is a short gene, spanning about 2 kb with two exons located on the long arm of chromosome 8 (8q13.1). The *CRH* gene encodes the 196 amino-acid long *CRH* precursor, which is then processed to the 41 amino-acid long CRH. It was first discovered in sheep by Vale et al. (1981). Rat and human CRH peptides are identical, whereas they differ from the ovine sequence only by seven amino acids.

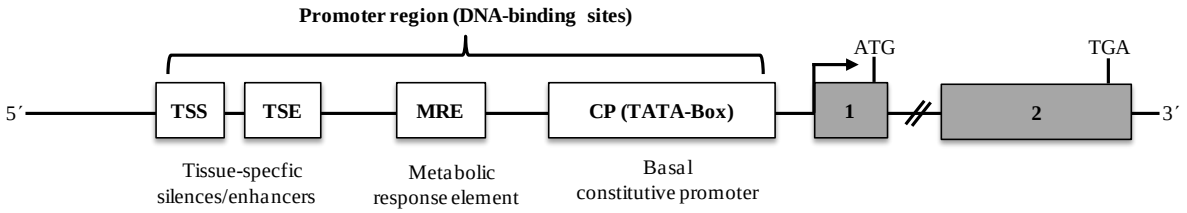

Figure 1.2 A schematic map of the human *CRH* gene with the promoter region. The grey rectangles represent transcribed exons. Introns are shown as lines. The transcription start site is indicated by the arrow. ATG and TAG are the positions of start and stop codons. Exon numbers are shown in Arabic numerals. (The location in the figure is not in relative correspondence to the actual sequence size.)

Functional polymorphisms and association studies

In the *CRH*, a set of four SNPs have been described, located in the 5' regulatory region of the *CRH* locus up to 4000 bp upstream of the transcription initiation site. The haplotypes of these four SNPs seem to be associated in reporter gene assays with different transcription levels (Wagner et al., 2006). This thesis focus on two of those SNPs, rs5030875 (T/G) and rs3176921 (T/C) (Table 1.4). The SNP rs3176921 (T/C) co-segregate with the SNPs rs5030877 and rs5030876 resulting in two alleles (A1/A2). The polymorphism rs5030875 (T/G) at the 5‘-regulatory region of the *CRH* locus (3371 bp before the transcription start site) correspond to alleles B1/B2. Finally, three different haplotypes could be assigned, haplotype 1 (T-T), haplotype

2 (G-C) and haplotype 3 (T-C). Haplotype 1 corresponds to haplotype A1B1, haplotype 2 to A2B2 and haplotype 3 to A2B1 in the study of Wagner and colleagues (2006).

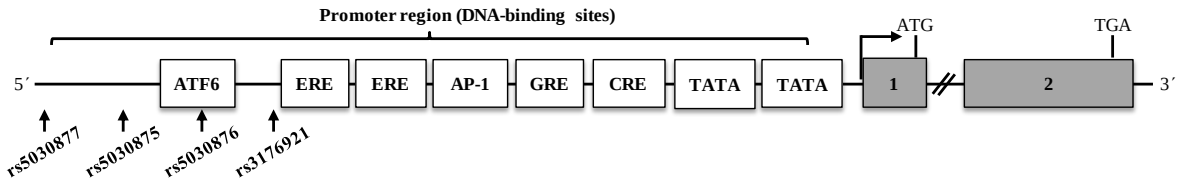

Figure 1.3 Location of investigated *CRH* SNPs in the promoter region and diagrammatic representation of the *CRH* promoter showing the sequential occurrence of response elements that are thought to have a functional role regulating gene expression. The grey rectangles represent transcribed exons. Introns are shown as lines. The transcription start site is indicated by arrows. ATG and TAG are the positions of start and stop codons. Exon numbers are shown in Arabic numerals. AF6: activating transcription factor 6. ERE: estrogen response element; AP-1: Activator protein 1; GRE: Glucocorticoid response element. CRE: cAMP response element; TATA: TATA box consensus sequence is TATAWAW. (The location in the figure is not in relative correspondence to the actual sequence size.)

Table 1.4: Overview of *CRH* gene variants

name	db SNP reference	Nucleotide change	Amino acid change	Locations	References
CRH	rs5030875	A/C	-	8:66181831	(Baerwald et al., 1999)
CRH	rs3176921	C/T	upstream variant 2KB	8:66179144	(Baerwald, Panayi, & Lanchbury, 1996)

Rs5030875 (T/G) and rs3176921 (T/C) SNPs of *CRH* have been used in a few association studies (Baerwald et al., 1999; Baerwald et al., 1996; Wagner et al., 2006). The minor G allele of rs5030875 was associated with higher stress-induced HPA axis function in patients with rheumatoid arthritis (Malysheva et al., 2011) but not with cortisol secretion or sensitivity in healthy men (Rosmond et al., 2001). The minor C allele of rs3176921 was associated with higher verbal intelligence and cognition values in healthy adults (Strohner, 2011) but not investigated concerning stress. So far, only one study analysed SNPs of *CRH* together with blood pressure, heart rate or cortisol response after stress testing. The rare allele of rs5030875 showed a significant interaction with a functional SNP (*ThtIII*I) of the *GR*, but no effects on

diurnal saliva cortisol variation or dexamethasone suppression (Rosmond et al., 2001). An overview of previous studies on rs5030875 and rs3176921 including associations with the HPA axis is given in Table 1.5.

Table1.5: An overview of previous studies on genetic variants of *CRH* examined in this thesis and their correlations with the HPA axis activity by different tests.

rs number	Testing HPA axis	Sample size/cells	gender	Gene interaction	Ref.
rs5030875 (Minor allele G)	salivary cortisol secretion	284	Men	No	Rosmond et al., 2001
	CAR	N/A			
	CPT	N/A			
	TSST	N/A			
	DST	284	Men	No	Rosmond et al., 2001
	In vitro	PC-12 cells	N/A	Major allele related to higher promoter activity after incubation with 8-Br-cAMP	Wagner et al., 2006
	In vitro	AT-20 cells	N/A	Major allele related to higher promoter activity after incubation with 8-Br-cAMP	
rs3176921 (Minor allele C)	CAR	N/A			Wagner et al., 2006
	CPT	N/A			
	TSST	N/A			
	DST	N/A			
	In vitro	PC-12 cells	N/A	Major allele related to higher promoter activity after incubation with 8-Br-cAMP	
	In vitro	AT-20 cells	N/A	Major allele related to higher promoter activity after incubation with 8-Br-cAMP	

1.2.2.2 Glucocorticoid receptor (*GR*)

In humans, the glucocorticoids (GC) regulate a broad spectrum of physiological functions, which are essential for life and play a vital role in the maintenance of basal the stress-related homeostasis (Chrousos, 2004). At the cellular level, the actions of the glucocorticoids are mediated by the glucocorticoid receptor (GR, gene symbols *GR*, *NR3C1*). The isoforms and levels of the GR protein throughout the HPA axis tissues control GC feedback and set the individual levels of stress reactivity and responsively. A complex interplay between genetic and epigenetic mechanisms regulates GR levels, protein isoforms, and potentially the endophenotype (Li-Tempel et al., 2016a).

Genetic architecture

GR is part of the steroid/thyroid/retinoic acid superfamily of nuclear receptors. It is a ligand-dependent transcription factor, regulating the expression of glucocorticoid-responsive genes positively or negatively. The human *GR* complementary DNA (cDNA) was isolated by expression cloning (Hollenberg et al., 1985). The *GR* is located in the long arm of chromosome 5 (q31.3). It comprises 9 exons. Exons 2-8 are core exons that are common to all protein isoforms. Additionally, two highly homologous receptor isoforms (α and β) are generated by alternative splicing of the *GR* in exon 9. In addition, and 5'-UTR of *GR* gene consists of 11 alternative splice variants (Leenen et al., 2016; Turner and Muller, 2005; Turner et al., 2014) (figure 1.4). The N-terminal domain (NTD) of the receptor is encoded by exon 2, while small exons 3 and 4 encode each of the two zinc fingers of the DNA-binding domain (DBD). The ligand-binding domain (LBD) is encoded by exons 5 - 9. The NTD is the most variable region between different nuclear receptor family members. The NTD contains an autonomous transcriptional activation function AF-1 that contributes to constitutive ligand-independent receptor activation. The conserved DBD is responsible for the recognition of specific DNA sequences. The C-terminal LBD is a multifunctional domain that mediates ligand binding, homo- and heterodimerisation, interaction

with heat shock proteins (HSPs), co-repressors and co-activators, nuclear localisation and transcriptional activity (Huang, Chandra, & Rastinejad, 2010).

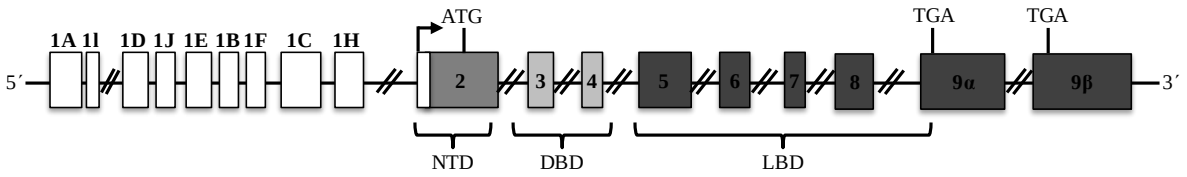

Figure 1.4: Genomic structure of the human *GR* is shown. Lines depict introns. Boxes depict exons. Filled boxes depict translated regions. White boxes depict untranslated regions. The transcription start site is indicated by the arrow. ATG and TAG are the positions of start and stop codons. Exon numbers are shown in Arabic numerals. Exon 1 is an untranslated region (UTR), exon 2 codes for NTD, exon 3 and 4 for DBD, and exons 5-9 for the LBD. Exons 1A–1I are alternatively spliced to a common acceptor site at the start of exon 2. Exons 1D--1H are the proximal CpG islands. DBD; DNA-binding domain; LBD: Ligand-binding domain; NTD: N-terminal domain. (The location in the figure is not in relative correspondence to the actual sequence size.)

Functional polymorphisms and association studies

In the *GR*, several SNPs have been extensively investigated for associations with the HPA axis function. Here, we examined the functional important SNPs rs10052950 (*TthIII*l), rs10482605 (NR3C1-1), rs10482614 (1H) rs6189 (E22E), rs6190 (R23K), rs41423247 (*Bcl*I), and rs6198 (9beta) (Figure1.5).

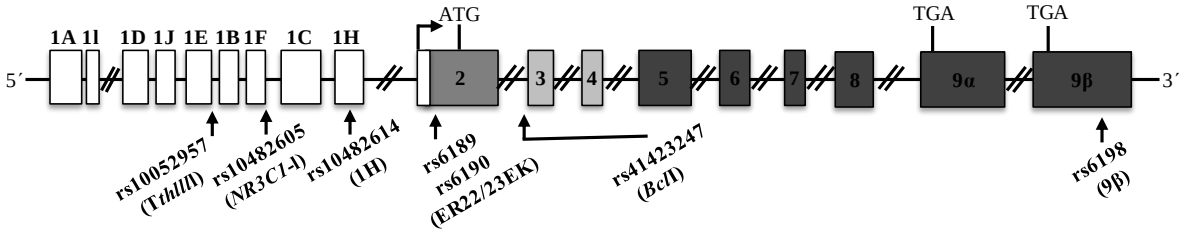

Figure 1.5. Location of the studied SNPs of the *NR3C1* gene (The location in the figure is not in relative correspondence to the actual sequence size.)

Table 1.6: An overview of *GR* (*NR3C1*) variants

name	db SNP reference	Nucleotide change	Amino acid change	Locations	References
*TthIII*I	rs10052957	C/T	-	Promoter 1D	(Detera-Wadleigh et al., 1991)
NR3C1-1	rs10482605	T/C	-	Promoter 1C	(van West et al., 2006)
1H	rs10482614	G/A		Promoter 1H	
E22E	rs6189	G/A	-	Exon 2	(van Rossum & Lamberts, 2004)
R23K	rs6190	G/A	arginine to lysine	Exon 2	(Koper et al., 1997)
*Bcl*I	rs41423247	C/G	-	Promoter Exon 3	(Murray et al., 1987)
9beta	rs6198	A/G	-	Exon 9	(Derijk et al., 2001)

Previously, the *TthIII*I polymorphism has been reported to be associated with higher basal cortisol levels in males (Rosmond et al., 2000). The SNPs ER22/23EK, NR3C1-1 and 9beta were associated with relative GC resistance and higher baseline cortisol levels (Kumsta et al., 2007; Manenschijn et al., 2009; van Rossum & Lamberts, 2004). When peripheral blood mononuclear cells (PBMCs) from healthy subjects were treated with dexamethasone for 24 hours in an *ex-vivo* setting, it has been shown that subjects homozygous for the minor C allele of *Bcl*I had higher *GR* mRNA levels than the subjects homozygous for the G allele (Xiang and Marshall, 2013). In line with this study, *Bcl*I has been found to be associated with lower morning cortisol levels after dexamethasone administration *in vivo* (Stevens et al., 2004). The 9beta polymorphism influences the ratio of GRalpha: GRbeta *in vitro* (DeRijk et al., 2001). Also, 9beta affects sensitivity to dexamethasone in males (Kumsta et al., 2007). An overview of previous studies on genetic variants in the human *GR* gene including associations with the HPA axis is given in Table 1.7.

Table 1.7: An overview of previous studies on genetic variants of *GR* examined in this thesis is shown, as well as correlations of *GR* variants with the HPA axis activity by different tests.

SNPs	Rs number	Testing HPA axis	Sample size/cells	gender inter-action	Gene interaction
T*thIII*I	**rs1005295 (Minor allele T)**	Profile	284 men	N/A	TT homozygotes showed higher total and evening cortisol levels (Rosmond et al., 2000)
NR3C1-1	**rs10482605 (Minor allele C)**	DST	219	yes	C allele is associated with higher post DST cortisol in men but with lower levels in women (Kumsta et al.,2008)
		TSST	219	yes	Male minor allele performed higher total cortisol (Kumsta et al., 2008)
		In vitro	brain cell lines	N/A	Minor allele showed reduced transcriptional activity (Kumsta et al., 2009)
1H	**rs10482614 (Minor allele G)**	DST	N/A	N/A	
		TSST	N/A	N/A	
		In vitro	N/A	N/A	
ER22/23 EK	**rs6189/90 (Minor allele A)**	DST	490	No	No (Van Rossum et al., 2006)
		TSST	206	No	No (Kumsta et al., 2007)
		In vitro	COS-1	N/A	Transcriptional activity in minor allele carriers is decreased (Russcher H et al.,2005)
***Bcl*I**	**rs41423247 (Minor allele G)**	DST	216	no	GG carriers showed lower post-dexamethasone cortisol (Stevens et al., 2004)
		DST	206	yes	Male GG carriers showed the highest CAR peak after DST (Kumsta et al., 2007)
		TSST	206	yes	Male GG carriers displayed the lowest saliva cortisol and lowest total cortisol (Kumsta et al., 2007)
9beta	**rs6198 (Minor allele G)**	DST	206	yes	Male AG carriers showed the highest CAR peak after DST (Kumsta et al., 2007)
		TSST	206	yes	Male AG carriers showed the highest total cortisol response (Kumsta et al., 2007)
		GSST	553 adolescents	no	No (Bouma et al.,2011)
		In vitro	COS-1	N/A	Minor allele increased the stability of GRbeta mRNA (DeRijk et al., 2001)

1.2.2.3 Mineralocorticoid receptor (*MR*)

The mineralocorticoid receptor (MR, gene symbols *MR*, *NR3C2*), also belongs to the nuclear receptor superfamily, functioning as a ligand-dependent transcription factor that mediates the effects of aldosterone on a variety of target tissues. MR has an identical affinity for mineralocorticoids as for glucocorticoids, which suggests that it functions as a high-affinity glucocorticoid receptor as well.

Genetic architecture

The *MR* gene is located in the long arm of chromosome 4 (q31.23) and consists of nine exons. The molecular mass of the deduced 984-amino acid protein is 107 kD. Zennaro et al. (1995) identified alternative splicing of the h*MR* gene in exon 1, termed α and β. The NTD of the receptor is encoded by exon 2, whereas small exons 3 and 4 both encode the two zinc fingers of the DBD. The LBD is encoded by exons 5 -9.

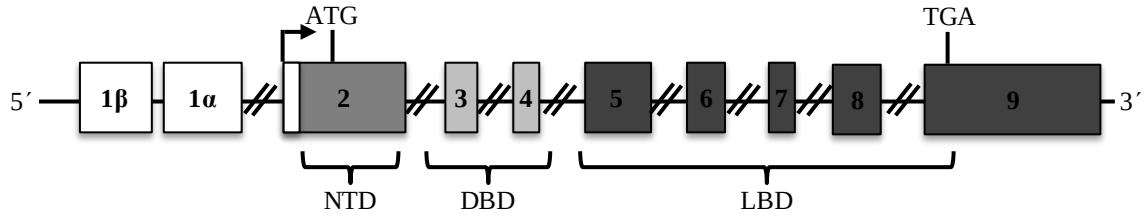

Figure 1.6: Genomic structure of the *MR* is shown. Lines depict introns. Boxes depict exons. Filled boxes and white boxes indicate the translated regions of the exons and the untranslated regions of the exons, respectively. The transcription start site is indicated by the arrow. ATG and TAG are the positions of start and stop codons. Exon numbers are shown in Arabic numerals. NTD: N-terminal domain; DBD: DNA-binding domain; LBD: Ligand-binding domain.

Functional polymorphisms and association studies

The role of two functional *MR* gene variants in HPA axis regulation and the CAR has been examined previously: The G/C variant located two nucleotides upstream the start codon in exon 1 (rs2070951, MR-2G/C) and the G/A variant at codon 180 (rs5522, MRI180V).

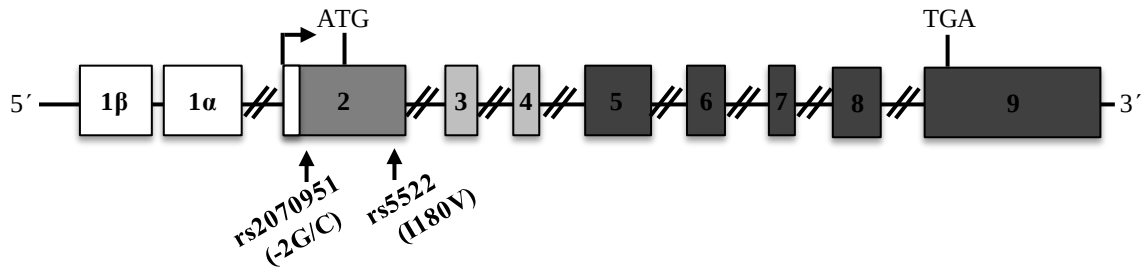

Figure 1.7: Location of the studied SNPs of the *MR* gene. (The location in the figure is not in relative correspondence to the actual sequence size.)

Table 1.8: Overview of *MR* (*NR3C2*) gene variants

name	db SNP reference	Nucleotide change	Amino acid change	Locations	References
*MR*_I180V	rs5522	A/G	Valin-Isoleucin	4:148436323Exon 2	(DeRijk et al., 2006)
*MR*_2G/C	rs2070951	G/C	In Kozak translation regulatory	4:148436862Exon 2 5'UTR	(van Leeuwen et al., 2010)

Kuningas et al. reported that carriers of the minor C allele of *MR*-2G/C had lower basal plasma cortisol levels (Kuningas et al., 2007). In addition, the homozygous G allele of *MR*-2G/C was associated with a higher CAR in men but not in women after dexamethasone administration (Van Leeuwen et al., 2010). After cortisol treatment, the minor G allele of *MR*I180V showed a loss of function in vitro. Furthermore, the minor G allele of *MR*I180V was associated with a lower CAR in men after dexamethasone administration (Van Leeuwen et al., 2010). An overview of previous studies on genetic variants in the human *MR* gene including associations with the HPA axis is given in Table 1.9.

Table 1.9: An overview of previous studies on genetic variants of *MR* examined in this thesis is shown, as well as correlations of *MR* variants with the HPA axis activity by different tests.

SNPs	rs number	Testing HPA axis	Sample size/cells	gender inter-action	Gene interaction	Ref.
***MR*I180V**	**rs5522 (Minor allele G)**	CAR	218	No	No	(van Leeuwen et al., 2010)
		DST	218	Yes	Male AA carriers showed higher CAR compared to male AG allele carriers	(van Leeuwen et al. ,2010)
		TSST	110	No	Minor allele showed higher cortisol	(DeRijk et al., 2006)
		In vitro	CV-1	N/A	*MR*180V allele revealed a mild loss of function using cortisol as a ligand	(DeRijk et al., 2006)
		Profile	563 older	No	C-allele carriers had lower cortisol levels than noncarriers	(Kuningas et al., 2007)
***MR*-2G/C**	**rs2070951 (Minor allele C)**	CAR	218	No	No	(van Leeuwen et al., 2010)
		DST	218	Yes	Female GG carriers showed the largest CAR	(van Leeuwen et al., 2010)
		GSST	553 adolescents	No	No	(Bouma et al., 2011)
		In vitro	COS-1	N/A	Minor allele less active *in vitro*	(van Leeuwen et al., 2010)

1.2.2.4 Serotonin transporter (5-*HTT*)

A key regulator of the serotonin (5-hydroxytryptamine) levels within the synaptic cleft is the serotonin transporter (5-HTT, gene symbols 5-*HTT*, *SLC6A4*), which actively mediates reuptake and recycling of released serotonin following neuronal stimulation. Therefore, the serotonin transporter represents a candidate that plays a role in the early CRH release from pituitary neurons and the subsequent cortisol release (Zhang et al., 2002) and finally determines the basal activity of the HPA axis.

Genetic architecture

The 5-*HTT* gene is located in the long arm of chromosome 11 (q11.2) and consists of 14 exons. The deduced 984-amino acid protein has a calculated molecular mass of 107 kD.

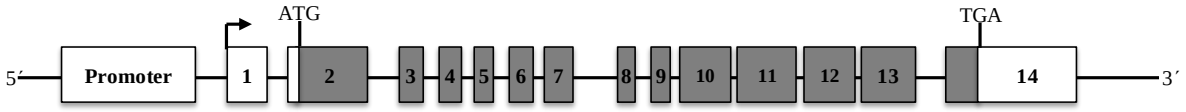

Figure 1.8: A schematic map of the human 5-*HTT* gene with the promoter region. Lines depict introns. Boxes depict exons. Filled boxes and white boxes indicate the translated regions of the exons and the untranslated regions of the exons, respectively. Exon numbers are shown in Arabic numerals. Arrow indicates the transcription start site. ATG and TGA mark the positions of the translational start and stop codons, respectively.

Functional polymorphisms and association studies

A SNP (rs25531), which is composed of adenine to guaninechange at sixth nucleotide (A/G), locates immediately upstream of the 5-hydroxytryptamine-transporter-linked polymorphic region (5-*HTTLPR*) (Kraft et al. 2005; Wendland et al., 2006; Zalsman et al., 2006). Both promoter variants of the serotonin transporter gene, 5-*HTTLPR* and SNP rs255331, enable four possible haplotypes: The long (L) allele with either A or G of rs255331, and the short (S) allele, also with A or G. Only the A allele of rs255331 in combination with the L allele of 5-*HTTLPR* (LA) yields high levels of 5-*HTT* mRNA, in contrast to the three other possible combinations LG, SA and SG (Wendland et al., 2006). Caspi and colleagues showed that the short allele was associated with

a higher incidence of depression after psychosocial stress (Caspi et al., 2003), although a recent meta-analysis could not confirm this initial finding (Risch et al., 2009). Figure 1.9 shows a graphical representation of the 5-*HTTLPR* in the serotonin transporter gene. An overview of previous studies on rs25531 and 5-*HTTLPR* including associations with the HPA axis is given in Table1.10.

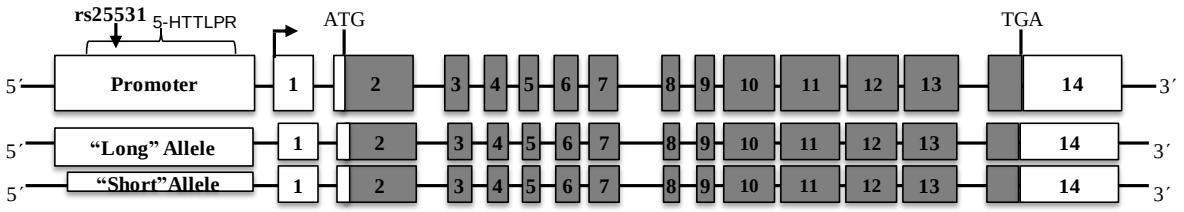

Figure 1.9: Allelic variation of the 5-*HTT*, including the serotonin-transporter-gene-linked polymorphic region (5-*HTTLPR*) and rs25531 SNP. Long and short refer to the presence or absence of the 43bp insertion/deletion polymorphism in the transcriptional control region. (The location in the figure is not in relative correspondence to the actual sequence size.)

Table 1.10: An overview of previous studies on genetic variants in the human 5-*HTT* examined in this thesis, and their correlation with the HPA axis activity by different tests.

rs number	Testing HPA axis	Sample size/cells	gender interaction	Gene interaction	Ref.
rs 25531 (Minor allele S/G)	CAR	216	yes	SS genotype showed the highest CAR in females, while showed the lowest CAR in males	(Wüst et al., 2009)
	DST	216	yes	No	
	TSST	216	yes	No	
	In vitro	lymphoblast cell line	N/A	The short variant polymorphism reduces transcriptional efficiency	(Lesch et al., 1996)

1.2.2.5 Brain-derived neurotrophic factor (*BDNF*)

The brain-derived neurotrophic factor (BDNF), which is encoded by the *BDNF* gene, is involved in the growth, differentiation and survival of neurons and synapses (Huang & Reichardt, 2001). Especially, it is most active in brain areas that contribute to learning and memory (e.g. the cortex and the hippocampus). The basal, unstimulated and unstressed HPA axis is further affected via indirect hippocampal input to the hypophysiotrophic neurons regulating the hypothalamic *CRH* gene expression. The neuro-plasticity of the hippocampus and the development of feedback mechanisms on the HPA axis are modulated via the BDNF. Jeanneteau et al. found that BDNF and glucocorticoid signaling regulate hypothalamic CRH expression via a unique mechanism involving cyclic adenosine monophosphate (cAMP) response-element binding protein (CREB) and its coactivator protein (Jeanneteau et al., 2012).

Genetic architecture

The human *BDNF* was cloned (Jones and Reichardt, 1990). It encodes a deduced 247 preproprotein that is proteolytically processed into a mature 119-amino acid protein. The *BDNF* gene is located on the long arm of chromosome 11 (q14.1) and consists of 11 exons (Pruunsild et al., 2007).

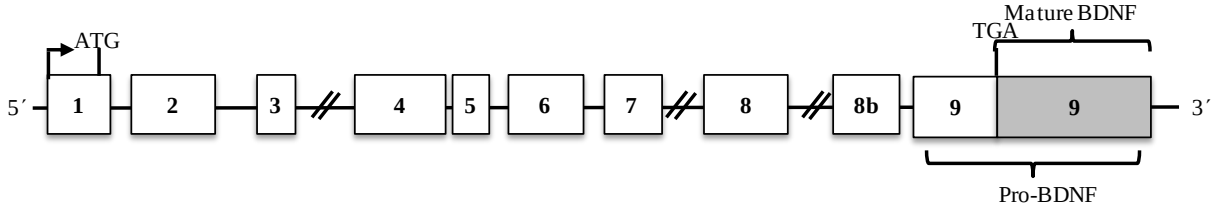

Figure 1.10: A schematic map of the human *BDNF* gene. Lines depict introns. Boxes depict exons. Vertical dashed lines indicate alternative splicing sites for the respective exons. Exon numbers are shown in Arabic numerals. Arrow indicates the transcription start site. ATG and TGA mark the positions of the translational start and stop codons, respectively.

Functional polymorphisms and association studies

A functional (G/A) SNP (rs6265) in the *BDNF* gene, which results in the conversion of the amino acid valine into methionine (Val66Met) (Egan et al., 2003) is intensively studied. This thesis is also focused on this SNP. The Met allele has been shown to be associated with poorer episodic memory, abnormal hippocampal activation assayed with functional magnetic resonance imaging (fMRI), and lower hippocampal N-acetyl aspartate, assayed with MRI spectroscopy (Egan et al., 2003). Neurons transfected with M66-BDNF-GFP (green fluorescence protein) showed lower depolarisation-induced secretion, while constitutive secretion was unchanged (Egan et al., 2003).

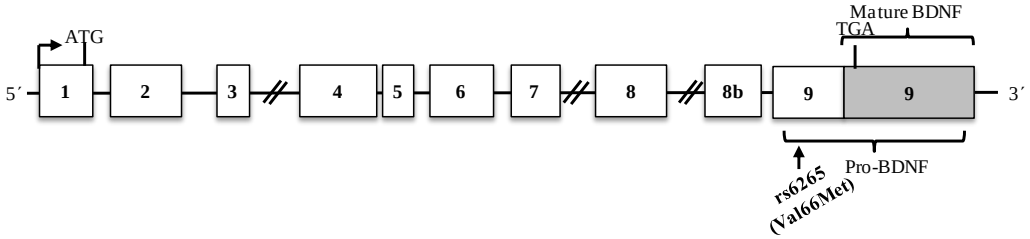

Figure 1.11: Location of the studies SNPs of the *BDNF* gene. (The location in the figure is not in relative correspondence to the actual sequence size)

The Met allele results in lower expression of the BDNF protein than the Val allele (Bath and Lee., 2006). In addition, it has been found to be associated with reduced hippocampal volume in healthy people (Szesko et al., 2005; Bueller et al., 2006) and in patients with major depression (Frodl et al., 2007). The Met allele is also associated with impaired memory in healthy people (Hariri et al., 2003; Pezewas et al., 2004). An overview of previous studies on rs6265 of *BDNF* including associations with the HPA axis activity is given in Table 1.11.

Table 1.11: An overview of previous studies on genetic variant rs6265 in *BDNF* examined in this thesis, and its correlation with HPA axis activity by different tests

rs number	Testing HPA axis	Sample size/cells	gender inter-action	Gene interaction	Ref.
rs6265 (Minor allele A)	TSST	100 men	no	Met allele carriers showed lower saliva cortisol level compare to subjects with the Val/Val genotype	(Alexander et al., 2010)
	In vitro	cultured neurons	N/A	Val/Met genotype impacted intracellular trafficking and activity-dependent secretion of BDNF	(Egan et al.,2003)

1.3 Scope and outline of the thesis

The present investigations examine the inter-individual variability of the stress response. The primary aim was to find critical links between the HPA axis and the SAM system involved in stress responses. To this end, all studies described in this thesis were designed to gain insight into how genetic factors influenced stress responses and at which level (see Figure 1.12). Three topics guided the investigations: Study 1 examined the role of HPA-axis-related genes that influence stress regulation, focusing on physiological as well as psychological stress indicators. Following up on the findings that *GR* SNPs influence both physiological (eg. AUCi) and psychological parameters (eg. TICS scale “work overload”), study 2 examined influences of *GR* (regarding genetic variation as well as methylation level) on both the SAM system and the HPA axis stress reactivity. Study 3 analyzed modulations of the physiological stress response by promoter haplotypes of the corticotropin-releasing hormone encoding (CRH) gene *in vitro* and *in vivo*. Mainly, the experiments described in this thesis were designed to address three specific questions.

1) Are HPA-related genetic factors simultaneously associated with the stress response in the HPA axis as well as in the SAM system?

It is not fully clear yet whether the two major stress response systems, the HPA axis and the SAM system, should better be regarded as separate systems or one complex system. HPA axis-related genetic factors might be one possible way to detect this, in particular, if HPA axis-related genetic factors were associated with some parameters representing these two systems simultaneously.

2) How are GR sequence variants and promoter methylation linked to responses to stress by the HPA axis and by the SAM system?

DNA methylation is unique, as it is the only epigenetic mechanism that may regulate gene expression. Although the associations of *GR* promoter methylation with diseases, such as

depression (Palma-Gudiel et al., 2015) are well studied, there is very little evidence on how it influences the HPA axis and the SAM system. Moreover, there might be a complex interplay between *GR* sequence variants and promoter methylation controlling *GR* levels in responses of the HPA axis and the SAM system. To investigate associations between genetic variants and promoter methylation, the socially evaluated cold pressor test (SeCPT) was performed in a cohort of healthy adults. These results provide some initial insights into the influence of the *GR* sequence variants and promoter methylation in this both major stress response systems.

3) Do haplotypes of the *CRH* gene modulate the physiological stress response *in vitro* and *in vivo* in a corresponding manner?

The CRH is a crucial neuropeptide mediating both the HPA axis and the SAM systems at different anatomical levels. Promoter haplotypes of the *CRH* gene can be studied *in vitro* and *in vivo*. It is assumed that *CRH* haplotypes modulate *in vitro* promoter activity and *in vivo* stress responses. The aim was to detect whether *CRH* haplotypes resulting in relatively stronger promoter activation also were associated with parameters represented in both the HPA axis and the SAM system.

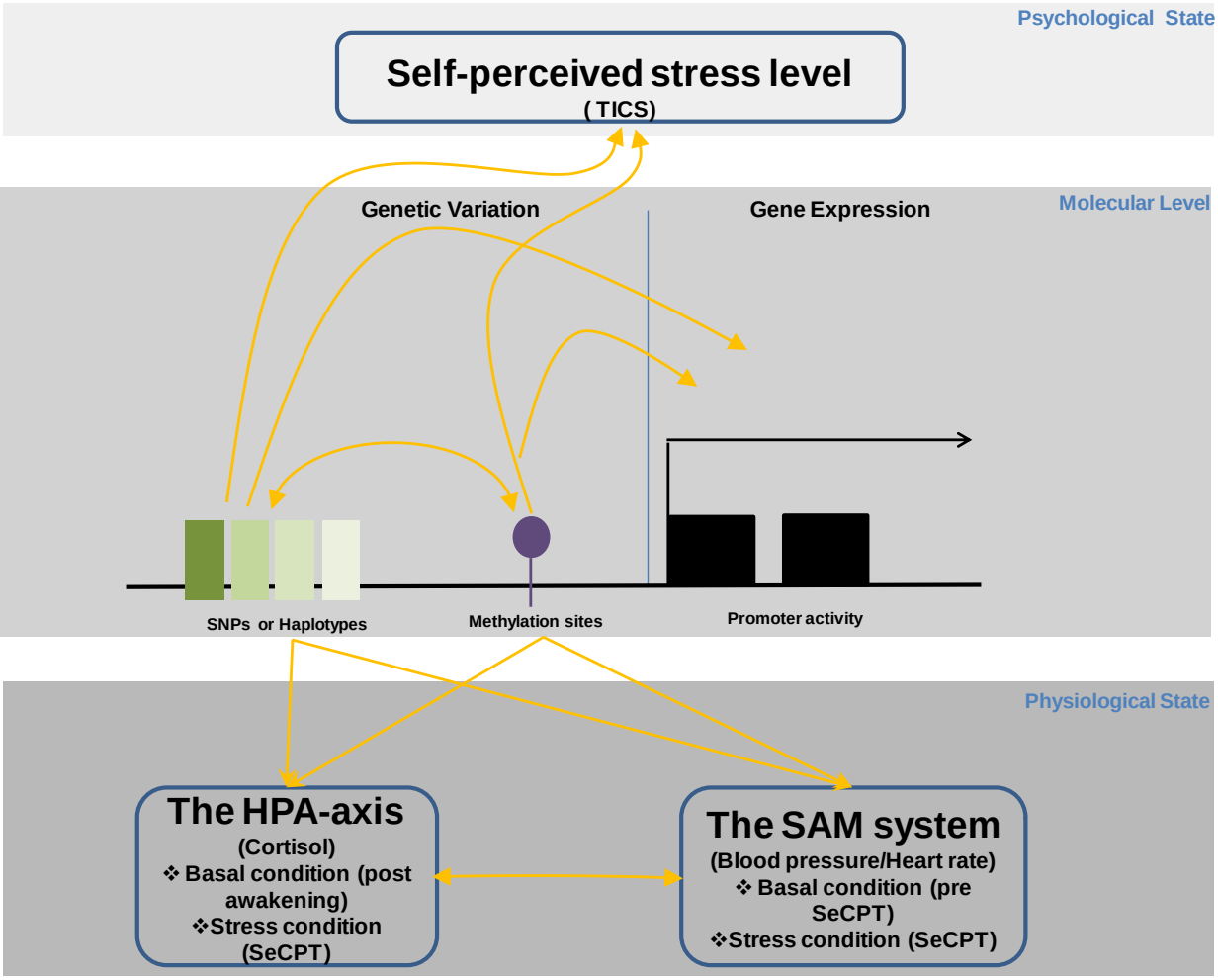

Figure 1.12: Schematic representation of the possible associations examined in this thesis. Three different levels are depicted: 1) Psychological state (tested by TICS questionnaire). 2) Molecular level [tested by stress-related genetic variation (checked by SNPs or haplotypes and methylation level) and gene expression (checked by promoter activity)]. 3) Physiological state (tested by the HPA axis and the SAM system). The HPA axis and the SAM systems are tested in basal condition and stress condition.

As **Chapter 1** outlines the general introduction, the following chapters 2 to 4 represents published studies and results of this thesis. **In Chapter 2**, the influence of polymorphisms of genes related to the HPA axis on the CAR as well as self-perceived stress is examined. On the molecular level, four examined genes are: the glucocorticoid receptor gene (*GR*), the mineralocorticoid receptor gene (*MR*), the serotonin transporter gene (5-*HTT*) and the gene encoding the brain-derived neurotrophic factor (*BDNF*). For detailed information see Figure 1.13.

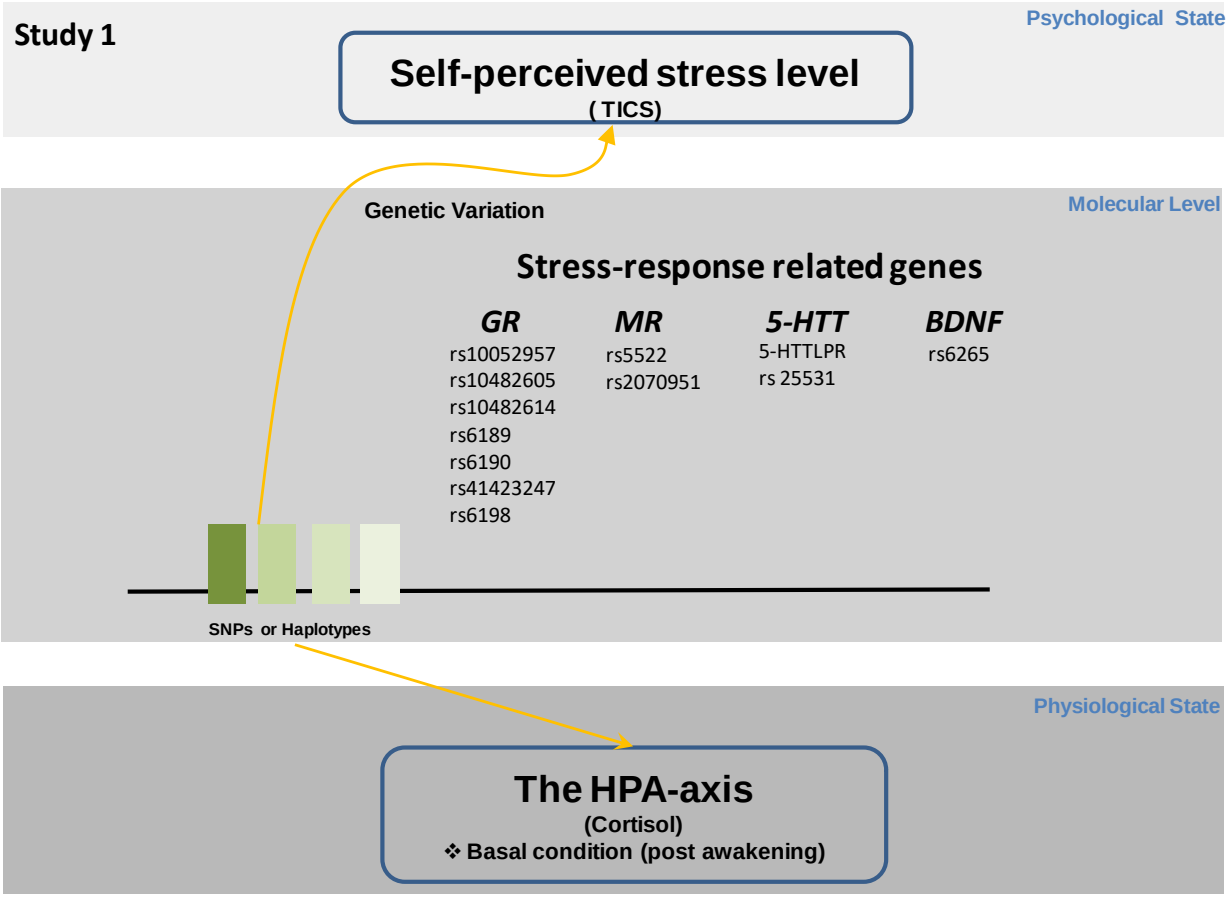

Figure 1.13: Schematic representation of the association examined in chapter 2.

Chapter 3 follows up on the finding that *GR* was linked most strongly to the HPA axis activity as well as psychological stress symptoms in Chapter 2. On the molecular level, seven genetic variants and promoter methylation of the *GR* gene are investigated. In the category of physiological state, the socially evaluated cold pressor test (SeCPT) is used for stress induction and cortisol levels are analyzed as the indicator for HPA axis activity during the SeCPT. Heart rate and blood pressure serve as indicators for the SAM system during the SeCPT. Detailed information is listed in Figure 1.14.

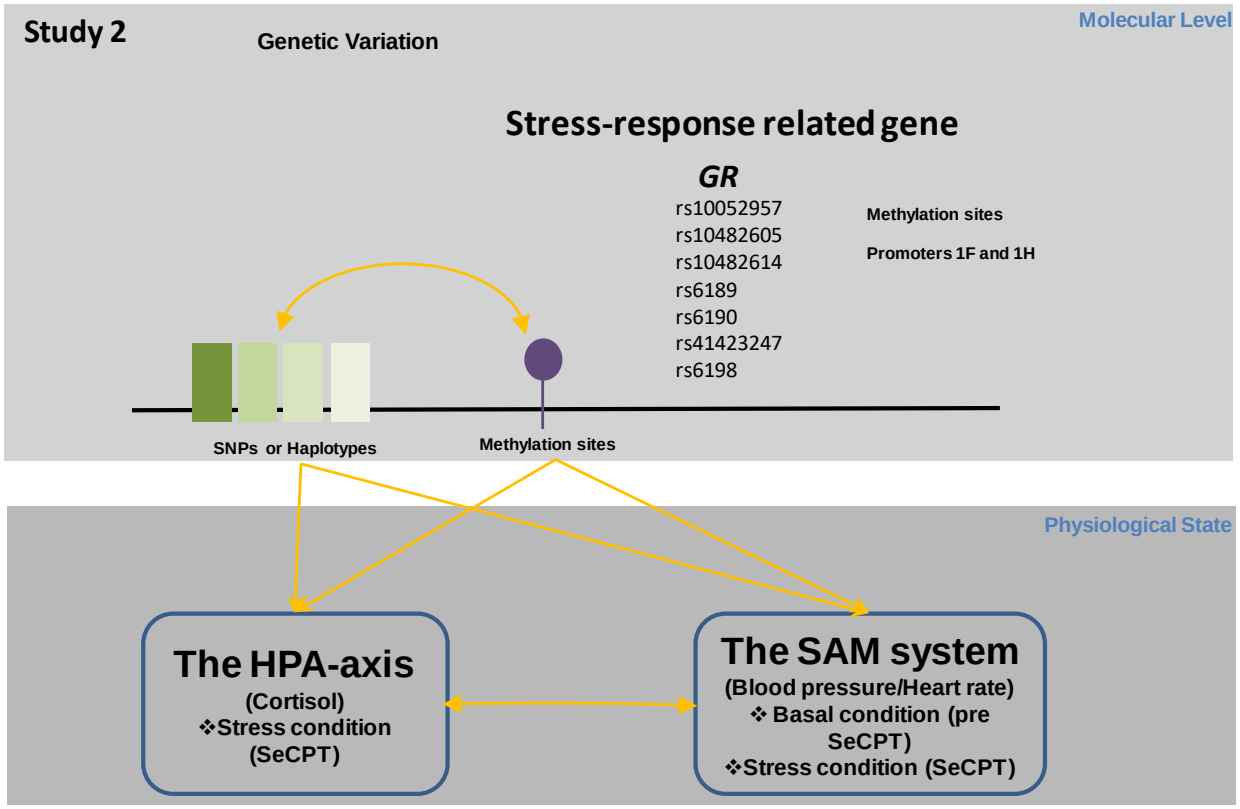

Figure 1.14: Schematic representation of the association examined in chapter 3.

Chapter 4 focuses on the molecular level, examining the *CRH* gene, which is encoding a key neuropeptide mediating the stress responses. In previous studies, genetic variants of the *CRH* promoter had been found to be associated with altered *CRH* promoter activity and physiological reactions. Assuming that *CRH* exerts indirect effects on the HPA axis, as well as direct effects on the SAM system (via binding of CRH receptors), functional characterisation of three CRH promoter haplotypes, is performed *in vitro* using a reporter gene assay under different stimulation conditions. In the category of physiological state, basal parameters such as post-awakening cortisol and blood pressure as well as stress reactivity measured after the SeCPT were investigated. Detailed information can be found in Figure 1.15.

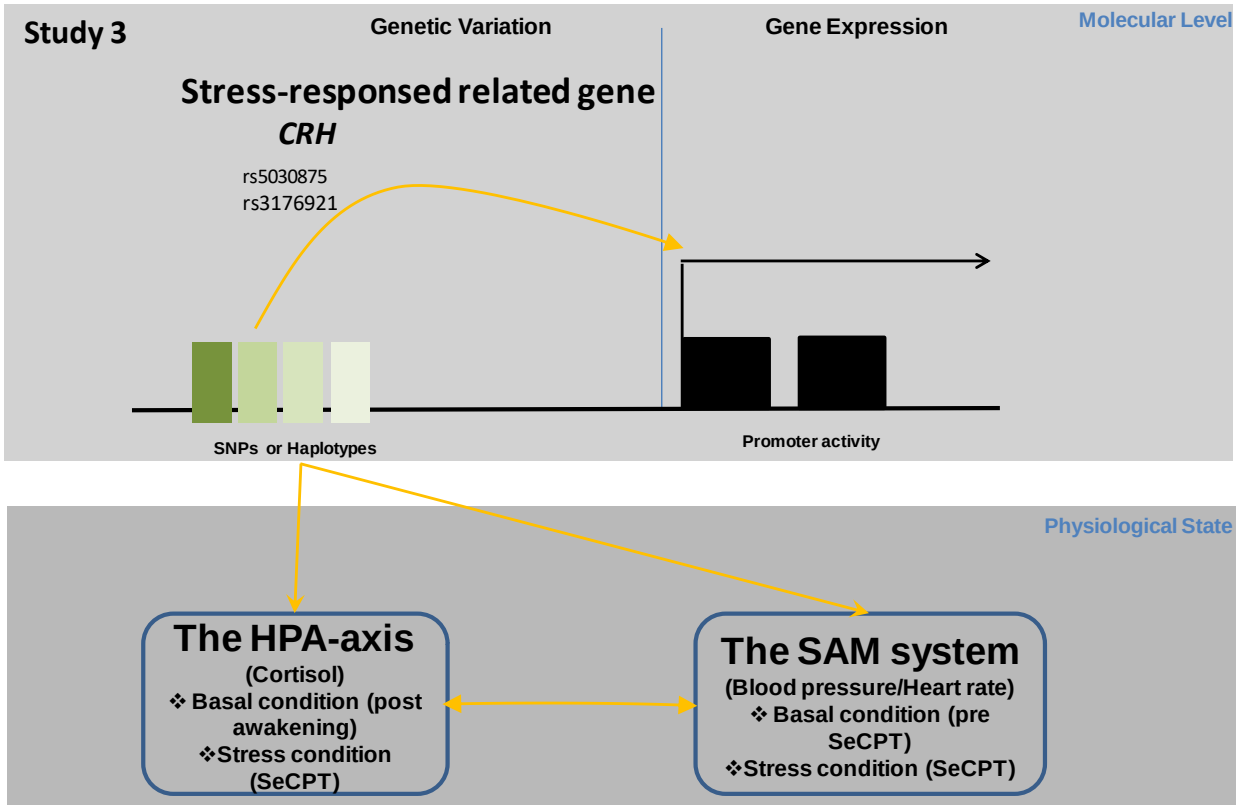

Figure 1.15: Schematic representation of the association examined in chapter 4.

Finally, in **Chapter 5** a synopsis of all major findings is presented and discussed. Directions for future research are suggested and practical implications are considered. **Chapter 6** contains a summary of the three studies.

Chapter 2

Polymorphisms of genes related to the hypothalamic-pituitary-adrenal axis influence the cortisol awakening response as well as self-perceived stress

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2.1 Abstract

The hypothalamic-pituitary-adrenal (HPA) axis is a crucial endocrine system for coping with stress. A reliable and stable marker for the basal state of that system is the cortisol awakening response (CAR). We examined the influence of variants of four relevant candidate genes; the mineralocorticoid receptor gene (*MR*), the glucocorticoid receptor gene (*GR*), the serotonin transporter gene (5-*HTT*) and the gene encoding the brain-derived neurotrophic factor (*BDNF*) on CAR and self-perceived stress in 217 healthy subjects. We found that polymorphisms of GR influenced both, the basal state of the HPA axis as well as self-perceived stress. MR only associated with self-perceived stress and 5-*HTT* only with CAR. *BDNF* did not affect any of the investigated indices. In summary, we suggest that *GR* variants together with the CAR and supplemented with self-reports on perceived stress might be useful indicators for the basal HPA axis activity.

2.2 Introduction

The inter-individual variability in the response to psychological or physical stress represents a central aspect of research in the field of neuroendocrinology. The hypothalamic-pituitary-adrenal (HPA) axis is one of the responsible endocrine systems that maintain physiological homeostasis after challenging situations. After stress, neurons in the hypothalamus release the corticotropin-releasing hormone (CRH), which subsequently leads to an increased secretion of cortisol from the adrenals. Disturbances in the HPA axis reactivity have been implicated in the pathogeneses of stress-related disorders such as posttraumatic stress disorder (Pacella, Feeny, Zoellner, & Delahanty, 2014) and depression (Plotsky, Owens, & Nemeroff, 1998).

The HPA axis activity could be targeted using different variables: first, detecting the daily profile as reflection of biological body rhythms; second, measuring the stress-induced activation, allowing conclusions on the negative feedback and/or third detecting the cortisol awakening response (CAR) as an indicator for the basal state of the HPA axis. The CAR is the distinct rise in cortisol levels within the first hour of awakening (Pajer, Gardner, Rubin, Perel, & Neal, 2001) and is influenced by psychosocial as well as genetic factors. A meta-analysis revealed associations of job stress and general life stress with increased CAR, and fatigue, burnout as well as exhaustion with reduced CAR (Chida & Steptoe, 2009). Additionally, several studies showed that specific genetic variants are associated with CAR (DeRijk, 2009), self-reported chronic stress (Feder, Nestler, & Charney, 2009), and stress-related personality traits (Bouchard Jr. & Loehlin, 2001; Bouchard, 2004). The CAR is highly individually stable, with a heritability of approximately 40% (Wüst, Federenko, Hellhammer, & Kirschbaum, 2000) and can be regarded as a stable biological marker of basal HPA axis activity. Measurable differences in the CAR can be considered as an “endophenotype” as it describes a pattern of the organism’s inner process (Gottesman, 2003). The CAR can be measured either as the area under the curve with respect to increase (AUCi) or as the area under the curve with respect to ground (AUCg). AUCi as the primary measure of the CAR represents the timely change of the dynamics of cortisol and is

related to the sensitivity of the system (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). AUCg corresponds to the overall level of cortisol released, which is depending on the baseline cortisol level at the time of awakening. AUCi has been considered to be a better indicator of the sensitivity or basal HPA reactivity than AUCg (Pruessner et al., 2003), since the HPA axis (re)activity is the responsiveness of this hormonal system, not a certain hormonal level.

The glucocorticoid (GC) sensitivity represents a key index for individual differences in HPA function (Désautés et al., 2002). Responses of the HPA axis exerted by cortisol are mediated by two hormone receptors, the mineralocorticoid receptor (MR, NR3C2; gene symbols *MR*, *NR3C2*; OMIM *600983) and the glucocorticoid receptor (GR, NR3C1; gene symbols *GR*, *NR3C1*; OMIM *138040). Both nuclear receptors trigger trans-activation and trans-repression of glucocorticoid target genes. The MR has a 10-fold higher binding affinity for GCs and maintains corticosteroid homeostasis by controlling basal HPA axis activity. Furthermore, MR is involved in the appraisal of novel situations and the selection of appropriate coping responses (DeRijk, De Kloet, Zitman, & Van Leeuwen, 2011). After a substantial increase of GCs, for example after awakening or acute stress (Bamberger, Schulte, & Chrousos, 1996), GR is activated and regulates its target genes. Therewith, GR mediates the regulation of genes involved in the immune response, metabolism and development and plays further an important role in stress-related behavior (DeRijk, Schaaf, & De Kloet, 2002). Genetic variants have been shown to mediate these processes via direct or indirect modulation of the HPA axis. In table SM-Table 1, an overview is given about them in our study investigated single nucleotide polymorphisms (SNPs) as well as length variations in relevant genes. Previously, two functional *MR* gene variants have been investigated with respect to HPA axis regulation and CAR, the G/C variant located two nucleotides upstream the start codon in exon 1 (rs2070951, *MR*-2G/C) and the G/A variant at codon 180 (rs5522, *MR*I180V). Kuningas and colleagues reported that carriers of the minor C allele of *MR*-2G/C had lower basal plasma cortisol levels (Kuningas et al., 2007). In addition, the

homozygous G allele of *MR*-2G/C was associated with a higher CAR following dexamethasone administration the previous day in men but not in women (Van Leeuwen et al., 2010). After cortisol treatment, the minor G allele of *MR*I180V showed a loss of function *in vitro.* Furthermore, the minor G allele of *MR*I180V was associated with a lower CAR following dexamethasone treatment in men (Van Leeuwen et al., 2010). In the *GR*, several SNPs have been extensively investigated for association with HPA axis function. Here, we investigated the functional important SNPs rs10052950 (*TthIII*l), rs10482605 (NR3C1-1), rs6189 (E22E), rs6190 (R23K), rs41423247 (*Bcl*I), and rs6198 (9beta). The *TthIII*l polymorphism was associated with higher basal cortisol levels in males (Rosmond et al., 2000). The SNPs ER22/23EK, NR3C1-1 and 9beta were associated with relative GC resistance and higher baseline cortisol levels (Kumsta et al., 2007; Manenschijn, Van den Akker, Lamberts, & Van Rossum, 2009; E. F. van Rossum & Lamberts, 2004). Subjects homozygous for the minor C allele of *Bcl*I had higher *GR* mRNA levels in peripheral blood mononuclear cells (PBMCs) treated *ex vivo* with dexamethasone compared to PBMCs derived from subjects homozygous for the G allele after 24 hours (Xiang & Marshall, 2013). In line with this study, *Bcl*I was associated with lower morning cortisol levels *in vivo* after dexamethasone administration (Stevens et al., 2004). The 9beta polymorphism influences the ratio of GRalpha: GRbeta *in vitro* (DeRijk et al., 2001). Furthermore, 9beta affects sensitivity to dexamethasone in males (Kumsta et al., 2007).

Besides the glucocorticoid-driven modulation of the HPA axis, serotonergic projections directly facilitate or inhibit HPA axis activity among other factors dependent on their topographically organization (Lowry, 2002). For example in the hypothalamus, serotonin activates corticotropin-releasing hormone (CRH) neurons as the initial impetus of the HPA axis leading to an increased release of CRH (Gold & Chrousos, 2002). Furthermore, the serotonergic system has been identified as being crucial for the prenatal development and early postnatal programming of the HPA axis (Andrews & Matthews, 2004). A key regulator of the serotonin levels within the synaptic cleft is the serotonin transporter protein (5-HTT, SLC6A4; gene symbols *5-HTT*,

SLC6A4, OMIM *182138), which actively mediates reuptake and recycling of released serotonin following neuronal stimulation. Therefore, the serotonin transporter represents a candidate that plays a role in the early CRH release from pituitary neurons and the subsequent cortisol release (Zhang et al., 2002) and finally determines the basal activity of the HPA axis. Previously, Wüst et al. investigated the impact of the promoter length polymorphism of *5-HTT*, 5-HTTLPR on the CAR and confirmed an influence of that variant on the CAR as an endophenotype in a gender-dependent manner (Wüst et al., 2009).

The basal, unstimulated and unstressed HPA axis is further affected via indirect hippocampal input to the hypophysiotrophic neurons regulating the hypothalamic CRH expression. The neuroplasticity of the hippocampus and the development of feedback mechanisms on the HPA axis are modulated via the brain-derived neurotrophic factor (BDNF, gene symbol *BDNF*; OMIM *113505) (Shalev et al., 2009). BDNF is essential for proper development and survival of e.g., serotonergic neurons and is involved in the BDNF dose-dependent decrease of the serotonin uptake (Autry and Monteggia, 2012). Jeanneteau et al. found that BDNF and glucocorticoid signaling regulate hypothalamic CRH expression via a unique mechanism involving cAMP response-element binding protein (CREB) and its coactivator protein, CRTC 2 (Jeanneteau et al., 2012). The most investigated variant of *BDNF*, a SNP (rs6265, Val66Met) in codon 66, has been shown to prevent the activity-dependent release of BDNF (Egan et al., 2003). Furthermore, subjects homozygous for the Met-encoding allele of the Val66Met polymorphism displayed higher ACTH and cortisol levels compared to carriers of the Val allele (Schüle et al., 2006). Therefore, we explored its potential contribution to the CAR and self-perceived stress. We hypothesize that variants of *GR* and 5-*HTT* affect both CAR and self-perceived stress. Whereas, we expect no effect of *MR* on the CAR (AUCi or AUCg) based on previous studies (Van Leeuwen et al., 2010), but we presume *MR* to influence self-perceived stress. Previously, the Trier Inventory for Chronic Stress (TICS) has been used as an index for stress-related HPA axis activity, although without considering genetic influences (Chida & Steptoe, 2009). Furthermore,

we assume gender-specific differences in the genetic associations due to previous reports on gender differences in HPA axis response patterns (Kajantie & Phillips, 2006; Uhart, Chong, Oswald, Lin, & Wand, 2006). In general, with regard to the scrutinized genetic influences on HPA axis activity, which is reflected by the sensitivity of this hormonal system, we expect to find influences of genotypes with regard to the AUCi rather than the alternative AUCg.

2.3 Methods

Participants

In total, the sample consisted of 231 participants from the University of Trier. Of them, 217 healthy men and women (115 females, mean age years = 23 ± 2.8; BMI 22.4 ± 0.25) completed the experimental procedures. Ethnic data were not collected, as population stratification was unlikely in our sample. Recently, Steffens et al. (2006) have reported a low extent of genetic heterogeneity in German samples. They detected only a slight north-south gradient with the difference being still several times smaller than those between samples drawn in Germany and samples in other Germanic populations, accounting for far less than 5% of the total variance. The present study was conducted in Trier, which is situated in middle Germany. The following exclusion criteria were applied: smoking more than five cigarettes per day, alcohol abuse, and any indication of circulatory disturbances or cardiovascular problems. Participants had to be medication free, contraceptives except ethinylestradiol and drospirenone containing ones such as Yasmin (Bayer-Schering Pharma, Leverkusen, Germany) were allowed at the time of testing as studies showed no influence of contraceptives on the CAR (Wüst et al., 2000). Furthermore, the participants were asked to indicate among others upcoming university exam, physical activities and sleep quality. The protocol was approved by the ethics committee of the state's medical association (*Landesärztekammer Rheinland-Pfalz*). All participants gave written informed consent.

Experimental protocol and psychological assessment

Participants were asked to collect saliva samples using Salivettes (Sarstedt, Nümbrecht) at home immediately after awakening and 15, 30, 45 and 60 min thereafter on two consecutive weekdays for the assessment of the CAR. They were instructed to refrain from food, drinks other than water and brushing their teeth before completion of saliva sampling. Furthermore and for reasons of compliance, they were told to pay special attention to keep the timing right to ensures reliability of their data. Salivary cortisol was analyzed in duplicate with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendörfer et al., 1992). The intra- and inter-assay coefficients of variance were 4.0-6.7% and 7.1-9.0% respectively (Macedo et al., 2008). 10 ml peripheral venous blood was taken into an EDTA-Monovette (Sarstedt, Nümbrecht) for DNA isolation. Additionally, participants filled out a short version of the German questionnaire on perceived chronic stress, the Trier Inventory for Chronic Stress [TICS; *Trierer Inventar zum chronischen Stress* (Schulz, Schlotz, & Becker, 2004)]. The TICS consists of nine subscales (*work overload, social overload, pressure to succeed, work discontent, excessive demands from work, lack of social recognition, social tensions, social isolation* and *chronic stress worrying*). Work overload, social overload, pressure to succeed can be grouped into stress from higher demands. The scales work discontent, excessive demands from work, lack of social recognition, social tensions, social isolation are all characterized by “deficits”. The third block contains only the chronic worrying. Moreover, information on gender, age, weight, anamnesis, contraceptives condition, alcohol weekly consumption and menstrual cycle were assessed by basic questionnaires.

DNA extraction and genotyping

DNA was extracted from 10 ml EDTA blood following a standard NaCl salting out method according to the protocol of Miller (Miller, Dykes, & Polesky, 1988). The *GR* polymorphisms

*TthIII*I (rs10052957), NR3C1-1 (rs10482605), E22E (rs6189), R23K (rs6190), *Bcl*I (rs41423247) and 9beta (rs6198) were genotyped using a single nucleotide primer extension reaction. For each SNP, specific primers were used in the SNPStart Master Mix kit from Beckman Coulter and fragments were analyzed with the CEQ8000 Genetic Analysis System (Beckman Coulter, Inc, Germany). *MR*-2G/C (rs2070951) and *MR*I180V (rs5522) were detected in duplicate using TaqMan pre-designed SNP genotyping assays, assay ID C12007869_20 and C1594392_10 respectively, in combination with TaqMan universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Sequencing of the PCR product was done with BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands) on an ABI Prism 3100-Avant Genetic Analyzer, according to the manufacturer's instructions. The Val66Met polymorphism (rs6265) of *BDNF* was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RELP) with 10 U of *Eco*72I for 2 h after initial PCR (Neves-Pereira et al., 2002). The 5-*HTT* length polymorphism (5-HTTLPR) was visualized after PCR amplification on a 2 % agarose gel and the SNP rs25531 via restriction with 10 U *MspI* for 2 h (Wendland, Martin, Kruse, Lesch, & Murphy, 2006).

Statistical analysis

GR genotyping was completed for *TthIII*I in a sample of 215 participants, NR3C1-1 for 214 participants, R23K for 215 participants and E22E, *Bcl*1 and 9beta for 217 participants. Genotyping of *MR*-2G/C, *5-HTT* and *BDNF* variants was completed for 217 participants, *MR*I180V for 216 participants.

Both promoter variants of the serotonin transporter gene, *5*-HTTLPR and SNP rs255331, enable four possible haplotypes: The long (L) variant of 5-HTTLPR with either A or G of rs255331, and the low active (S) variant, also with A or G. Only the A allele of rs255331 in combination with the L allele of *5*-HTTLPR (L_A) yields high levels of 5-HTT mRNA, in contrast to the three other possible combinations L_G, S_A and S_G (Wendland et al., 2006). Therefore, three "transcription

activity" (TA) groups were formed for statistical analysis with respect to promoter activity: 1, high/high (genotype L_A/L_A); 2, high/low (genotypes L_A/L_G, L_A/S_A); 3, low/low (genotypes L_G/L_G, L_G/S_A, S_A/S_A, S_A/S_G) (Chan et al., 2008). Individuals homo- or heterozygous for the Met-encoding allele of *BDNF* were compared to homozygous carriers of the Val-encoding allele (Neves-Pereira et al., 2002). *GR and MR* genotyping data were reduced by dichotymizing the SNPs with low minor allele frequencies (homozygous minor allele: n < 6) combining carriers of one or two minor alleles in one group. This has been applied for the *GR* SNPs NR3C1-1, 9beta and E22E and the MR SNP *MR*I180V. *F*or *TthIII*I and *Bcl*1, three groups (homozygous minor allele, homozygous major allele and heterozygous) were compared.

All SNPs were tested for Hardy-Weinberg equilibrium (HWE). Haploview 4.2 (Barrett, Fry, Maller, & Daly, 2005) was used to assess inter-marker linkage disequilibrium scores (LD scores), expressed as D' and r^2. Individual *GR* and *MR* haplotypes were reconstructed using the program PHASE, version 2.1 [(Stephens, Smith, & Donnelly, 2001) http://stephenslab.uchicago.edu/software.html#phase], which uses an algorithm based on coalescence-based Bayesian Haplotype inference for predicting haplotypes from genotype data, combining modeling strategy with computational strategies. The lowest observed haplotype probability was computed with 0.002%. No individual was excluded based on haplotype formation. Haplotype comparisons have been performed separately between all carriers of one haplotype and all non-carriers of the same haplotype. CAR levels assessed on two consecutive days were averaged in order to enhance the reliability of the measure (Hellhammer et al., 2007). The area under the curve with respect to ground (AUCg) and increase (AUCi) (Pruessner et al., 2003) served as dependent variables in the subsequent analyses of variance (ANOVA) with the two between-participants-factors genotype and gender. TICS subscale scores (*work overload*; *social overload*; *excessive demands from work*; *lack of social recognition*; *work discontent*; *social tensions*; *pressure to succeed*; *social isolation*; and *chronic stress worrying*) were used as dependent variables in separate ANOVAs with the two between-participants-factors gender and

genotype (with each SNP and each haplotype as the genotype factor). Results were considered statistical significant with a *p*-value < 0.05. The power calculations were performed with the software G-Power (Faul, Erdfelder, Lang, & Buchner, 2007). Given the sample size of n = 217, the statistical power was sufficient to detect a small-to-medium-size effect if alleles were equally distributed. However, some of the examined SNPs involved low-frequency alleles that imply that the given power might be only sufficient to detect large effects for the respective genetic variables. To correct for false positives because of multiple testing, we applied the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995), which allows a stepwise correction for type I errors. All results shown are the mean ±SEM unless otherwise stated. Statistical analyses were performed using SPSS 20.0.

2.4 Results

Genotype and haplotype distributions

We investigated six *GR* polymorphisms (*TthIII*I, NR3C1-1, E22E, R23K, *Bcl*I and 9beta), two *MR* polymorphisms (*MR*-2G/C and *MR*I180V), one 5-*HTT* SNP (rs25531), the length polymorphism (*5*-HTTLPR) of *5-HTT*, as well as the Val66Met polymorphism of *BDNF* (for details, see Table 2.1 and SM-Table 2.1). The SNP R23K (rs6190) deviated from the HWE and was therefore excluded from further analyses. Pairwise comparisons between the *GR* SNPs resulted in a high linkage disequilibrium (LD) for NR3C1-1 and E22E (D' = 1, r^2 = 0.14), NR3C1-1 and 9beta (D' = 1, r^2 = 0.87), 9beta and *Bcl*I (D' = 0.94, r^2 = 0.11) as well as for 9beta and *TthIII*I (D' = 0.97, r^2 = 0.39) (Figure 2.1). The five polymorphisms of *GR* constitute six haplotypes with frequencies above 3.0% (Figure 2.1), other estimated haplotypes had frequencies between 0.22% and 0.88%. The haplotype with the highest frequency (41.6%) will be referred to as Haplotype 1. The LD and the haplotype structure in our sample were similar to that previously reported (Cao-Lei et al., 2011). We did not find any difference of allele frequencies with respect to age and gender, with two exceptions: the frequencies of *Bcl*I genotypes differed significantly between men and

women. As a consequence, we found a comparable difference between *GR* haplotype 2 carriers and non-carriers, since the minor allele of *Bcl*I is part of this haplotype.

In line with the literature, both *MR* SNPs investigated here were found in LD (D' = 1, r^2 = 0.09). *MR* haplotype 1 (50%; see Figure 2.2) was the most prevalent haplotype. For the *MR* genotypes and haplotypes, there were no differences in the distributions with respected to age and gender.

The LL genotype of 5-HTTLPR was found in 67 cases and the genotypes LS and SS were detected in 106 subjects and 43 subjects, respectively, which is consistent with previously reported frequencies (Lesch et al., 1996). Neither for 5-HTTLPR (*p* = 0.09) nor for the 5-HTTLPR/rs25531 alleles (*p* = 0.25), genotype distributions deviated significantly from HWE. Subjects were assigned to the "transcriptional activity" (TA) group 1 (high/high, n = 54), TA group 2 (high/low, n = 111) or TA group 3 (low/low, n = 52). Likewise for *5*-HTTLPR/rs25531, there were no differences in the allele distributions with respected to age and gender.

The alleles constituting the Val66Met polymorphism of the *BDNF* did not deviate from the HWE (*p* = 0.12). Moreover, genotype frequencies (Val66Val 61.2%, Val66Met 34.6%, and Met66Met 4.2%) were similar to those reported in previous studies for Caucasian populations (Gatt et al., 2009). Individuals homo- or heterozygous for the Met-encoding allele of *BDNF* (n = 84) were compared to homozygous carriers of the Val-encoding allele (n = 133). There were no differences in the allele distributions with respected to age and gender for the Val66Met polymorphism.

Table 2.1: Descriptive data of single markers of four candidate genes, the mineralocorticoid receptor gene (*MR)*, the glucocorticoid receptor gene (*GR*), the serotonin transporter gene (5-*HTT*) and the gene encoding the brain-derived neurotrophic factor (*BDNF*) in 217 participants using Haploview

Gene	SNPs	position	obsHET	predHET	HWpval	% Geno	MAF	Alleles
GR	*TthIII*I	-142766894	0.465	0.464	1.0	99.1	0.365	C : T
	NR3C1-1	-142763714	0.356	0.336	0.518	98.7	0.213	T : C
	E22E	-142760532	0.075	0.072	1.0	100.0	0.037	G : A
	R23K	-142760530	0.084	0.105	0.0437	99.1	0.055	G : A
	*Bcl*I	-142758768	0.404	0.461	0.0787	100.0	0.360	C : G
	9beta	-142637814	0.307	0.306	1.0	100.0	0.189	A : G
MR	I180V	-149143647	0.177	0.161	0.301	99.1	0.088	A : G
	-2G/C	-149144186	0.476	0.5	0.5296	99.6	0.493	G : C
5-*HTT*	rs25531	-1724774511	0.088	0.084	1.0	100.0	0.044	A : G
BDNF	Val66Met	-1127608025	0.317	0.358	0.1218	100.0	0.233	G : A

Abbreviations: HWpval: p value for Hardy-Weinberg-Equilibrium; MAF: minor allele frequency; obsHET: observed heterozygosity; position: chromosomal location; predHET: predicted heterozygosity; SNP: single nucleotide polymorphism; % Geno: Genotyping frequency

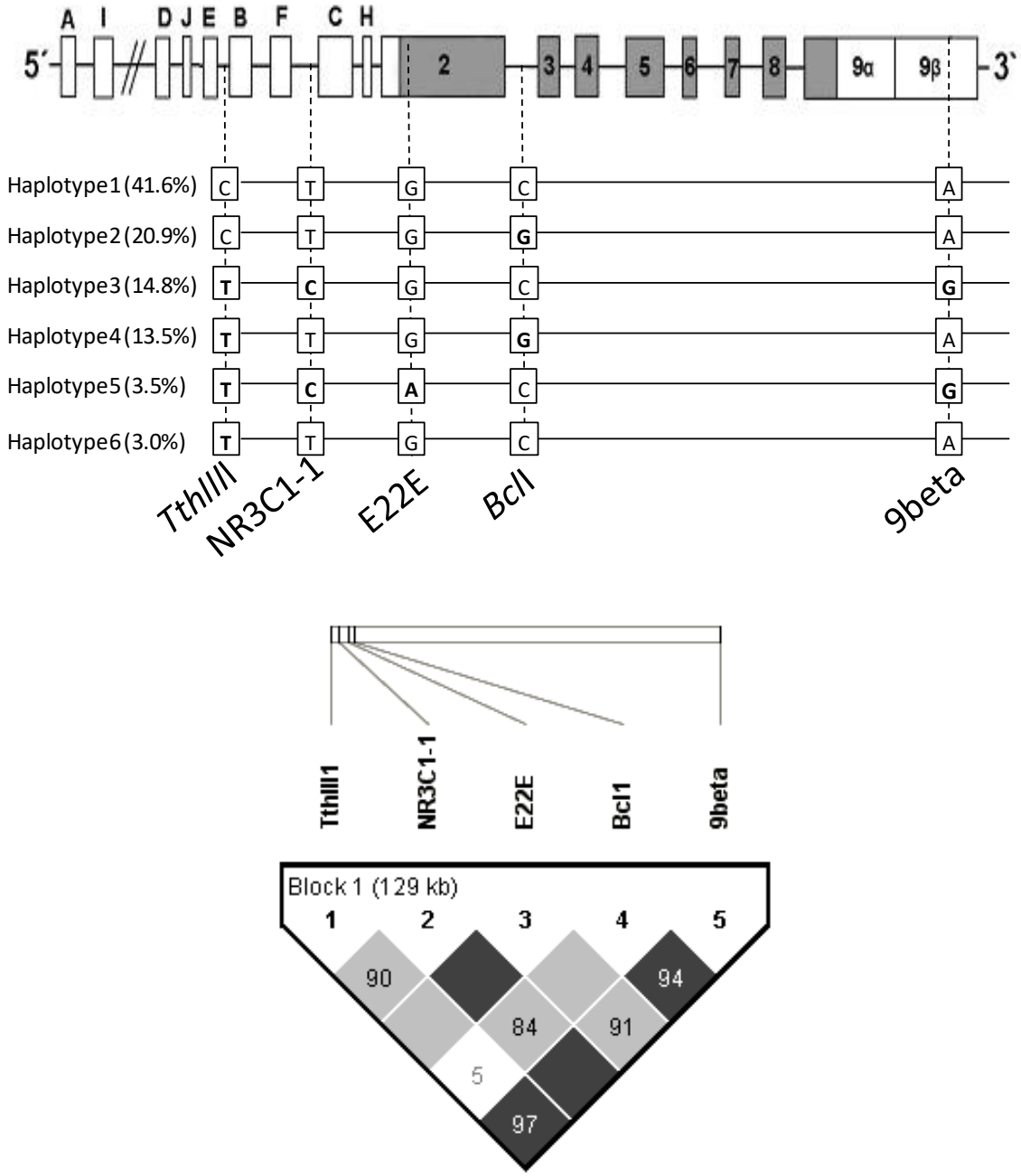

Figure 2.1: **Schematic overview of the *GR* gene polymorphisms and haplotype frequencies**

Genomic organization of the human glucocorticoid receptor gene (*GR*) is indicated in the upper part of the figure. Boxes indicate exons and the coding region of the gene is drawn in dark shade. The 5´ region of the gene shows multiple first exons (capital letters A–I). The middle part of the figure represents the observed haplotype structure and frequencies. Haplotypes with a frequency above 3% (calculated by Haploview from N = 217) are shown. The minor alleles are highlighted in bold letters. The lower panel shows the linkage disequilibrium (LD) confidence bounds color scheme pattern for the five *GR* SNPs using Haploview. Diamonds with dark grey color represent the strong evidence of LD; Diamonds with light grey color are uninformative; Diamonds with white color represent the strong evidence of recombination. Numbers within the LD diamonds represent the value of D prime (D'), which represents the correlation coefficient between two loci.

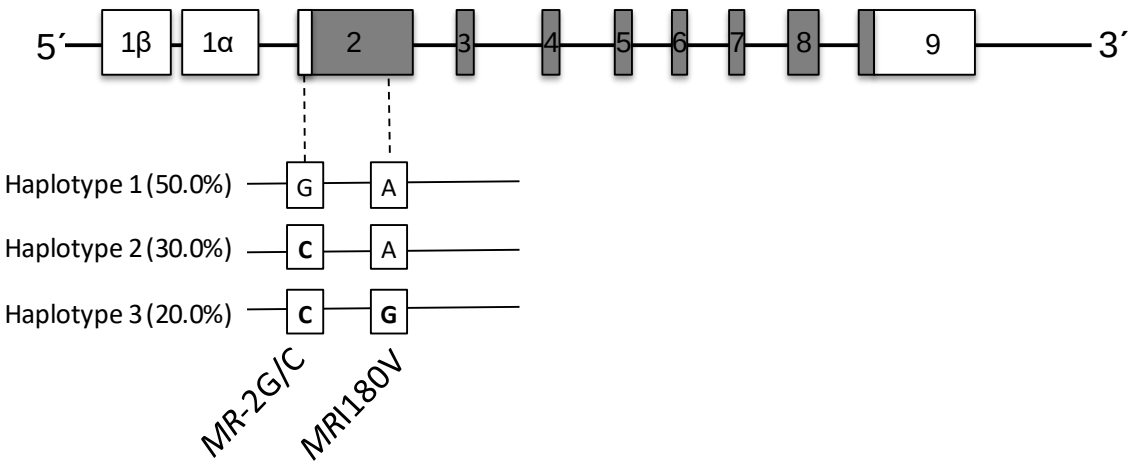

Figure 2.2: Schematic overview of the *MR* gene polymorphisms and haplotype frequencies

Genomic organization of the human mineralocorticoid receptor gene (*MR*) is indicated by the location of the *MR* SNPs *MR*-2G/C and *MR*I180V, the haplotype structure and frequencies of the haplotypes formed by these SNPs. Dark shade boxes represent exonic regions and the black line boxes represents the intronic regions of the gene. *MR*-2G/C is located in the untranslated region just 2 nucleotides before the translation start and *MR*I180V is located in the translated region of exon 2. The minor alleles are represented in bold letters. The percentages in brackets refer to the haplotype frequency observed in this cohort.

Associations of genes with the cortisol awakening response (CAR)

The influences of genotypes and gender on the CAR expressed as AUCi and AUCg were calculated using a two-factor ANOVA (Table 2.2, Figure 2.3). The two SNPs *TthIII*I and 9beta of *GR* significantly impacted AUCi. The interaction of gender and *TthIII*I was significant, ($F(2, 208) = 3.645$, $p = 0.028$), whereas the main effect of *TthIII*I was not ($F < 1$). Simple effects analyses revealed that men homozygous for the minor allele (T) of *TthIII*I showed a significantly higher AUCi than women homozygous for the minor allele ($p = 0.022$), whereas men and women homo- or heterozygous for the major allele (C) did not significantly differ ($p = 0.164$, $p = 0.699$). A similar result was obtained for 9beta with a significant interaction of gender and genotype [$F(1, 212) = 4.732$, $p = 0.031$]. Men, but not women, carrying the minor G allele showed marginally higher AUCi compared to men homozygous for the major allele (A) ($p = 0.088$).

We investigated possible interactions between GR haplotypes and gender with respect to AUCi by a two-factor ANOVA. Significant interactions were found for gender and haplotype 2 [F (1, 212) = 4.391, p = 0.037] and haplotype 3 [F (1, 212) = 5.865, p = 0.016] with regard to AUCi, whereas the main effects were not significant (F < 1). Simple effects analyses showed that male carriers of haplotype 2 displayed a significantly lower AUCi compared to male non-carriers (p = 0.018), whereas female carriers and non-carriers did not significantly differ (p = 0.553). The AUCi of male carriers of haplotype 3 was marginally higher than the AUCi of male non-carriers (p = 0.054), whereas female carriers of haplotype 3 and female non-carriers did not differ (p = 0.140). No other associations concerning *GR* SNPs approached significance, Fs < 1.95, p_{all} > 0.114.

We found a significant association of 5-*HTT* and the AUCi. There was a significant main effect of the genotype [F (2, 210) = 4.634, p = 0.011] but no significant interaction with gender [F (2, 210) = 2.663, p = 0.072] as reported by Wüst et al. (2009). The TA group 3 (low/low) had the lowest AUCi level compared to high/high and high/low groups.

There were no significant associations of *MR* and *BDNF* with the CAR AUCi measures in our study. Interestingly, none of the genes showed an association with the AUCg, which is supposed to correspond to a rather basal hormonal level.

Association of genes with TICS

To evaluate the influences of genotype and haplotype on chronic stress measured with the TICS, a series of between-participants ANOVAs with genotype or, respectively, haplotype and gender was performed (Table 2.2, Figure 2.3).

With regard to the “work discontent” scale, there was a significant interaction of gender and *TthIII*l [F (2, 207) = 3.554, p = 0.030]. Simple effects analyses showed that men homozygous for the minor T allele indicated lower “work discontent” scores compared to women homozygous for

this allele (p = 0.021). Significant interactions of gender with NR3C1-1 were observed for the TICS subscales “work overload” [F (1, 208) = 4.590, p = 0.033] and “social overload” [F (1, 208) = 4.078, p = 0.045]. Simple effects analyses showed that “work overload” and “social overload” scores were higher for men carrying the minor C allele compared to men homozygous for the major T allele (p = 0.045, p = 0.029). No such differences were found in women. Furthermore, we found a significant interaction of gender with *GR* haplotype 5 (see Fig 2.3) on the TICS subscales “work overload” [F (1, 211) = 10.871 p = 0.001], “excessive demands from work” [F (1, 211) = 3.925, p = 0.049] and “pressure to succeed” [F (1, 211) = 8.447 p = 0.004]. Simple effects analyses showed that male carriers of *GR* haplotype 5 had higher TICS scores “work overload” and “pressure to succeed” compared to female carriers (p = 0.005, p = 0.004). Female *GR* haplotype 5 non-carriers displayed higher “work overload” scores than male non-carriers (p = 0.039). Female *GR* haplotype 5 carriers showed lower “excessive demands from work” than female non-carriers (p = 0.015), whereas male carriers and non-carriers of *GR* haplotype 5 did not differ significantly (p = 0.501).

The *MR* I180V genotypes and *MR* haplotype 3 significantly influenced the “lack of social recognition” score [F (1, 210) = 5.546, p = 0.019; F (1, 211) = 5.659, p = 0.018.] Individuals heterozygous for *MR* I180V had a higher “lack of social recognition” score compared to those homozygous for the major A allele. Carriers of the *MR* haplotype 3 had higher “lack of social recognition” scores than non-carriers. With respect to the “social isolation” subscale and *MR* I180V, the main effect of genotype was significant [F (1, 210) = 3.896, p = 0.050]. Subjects heterozygous for the *MR* I180V had higher scores than homozygous carriers of the major A allele. “Work discontent” was influenced by a significant interaction of 5-*HTT* genotype and gender [F (2, 209) = 4.217, p = 0.016]. Further simple effects analyses revealed significant higher “work discontent” scores for homozygous female carriers of the low active allele compared to males (p = 0.036). Women in the high/low group displayed lower “work discontent”

than men (p = 0.055). No significant difference was observed between men and women homozygous for the high active allele (p = 0.618).

False-discovery-rate correction

Given the number of conducted statistical analyses, the possibility of false positive results needs to be taken into account. Altogether, we analyzed the influence of 18 genetic variables (alleles, haplotypes and genotypes) on two measures of the CAR and nine TICS scales. We tested for potentially false positives among the emerged significant results by applying the Benjamini-Hochberg procedure to correct for multiple testing (with a maximum false discovery rate of 0.20). We tried to maximize the control by simultaneously including all 18 analyzed genotype variables in the correction (*i.e.*, all SNPs plus all haplotypes) but we conducted separate corrections for main effects of these variables and gender x genotype interaction effects (because these independent statistical effects should be accounted for independently).Some of the significant results of the reported ANOVAs concerning TICS scales did not pass this additional test (see Table 2.2). Therefore, we assume that genetic impacts on self-reported stress as reflected by the TICS are, in fact, quite rare and we suggest interpreting such associations with caution, especially if correction for multiple statistical testing is not considered. In contrast, the significant effects of genetic variables on the CAR (AUCi) remained unaffected by the application of the Benjamini-Hochberg procedure. Thus, the associations of genetic variables with the CAR as a physiological measure might be more robust than the associations of genetic variables with subjective, psychological measures.

Table 2.2: The association of SNPs and Haplotypes of the four genes *GR*, *MR*, 5-*HTT* and *BDNF* with the CAR and TICS.

	***GR* rs1005295_TthIII/**		***GR* rs10482605_NR3C1-1**		***GR* rs6189_E22E**		***GR* rs41423247_*Bcl*I**		***GR* rs6198_9beta**		***GR* Haplotype 1 CTGCA**		***GR* Haplotype 2 CTGGA**		***GR* Haplotype 3 TCGCG**		***GR* Haplotype 4 TTGGA**		***GR* Haplotype 5 TCACG**		***GR* Haplotype 6 TTGCA**		***MR* I180V_rs5522**		***MR* -2G/C_rs2070951**		***MR* Haplotype 1 GA**		***MR* Haplotype 2 CA**		***MR* Haplotype 3 CG**		**5-HTTLPR & rs25531**		***BDNF* rs6265_Val66Met**	
	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]	maineffect[a]	interaction[b]
CAR																																				
AUCg																																				
AUCi																																				
Chronic stress (TICS)																																				
work overload				X																																
social overload				X																																
excessive demands from work																				X																
lack of social recognition																																				
work discontent		X																																		
social tensions																																				
pressure to succeed																																				
social isolation																																				
chronic stress worring																																				

Abbreviations: AUCg: The area under the curve with respect to ground; AUCi: The area under the curve with respect to increase; CAR: the cortisol awakening response. TICS: Trier Inventory for Chronic Stress; [a] genotype or haplotype as main effect; [b] interaction between gender and genotype or haplotype; *p* values are provided according to a color code as indicated by the appended scale.

	> 0.05
	0.01-0.05
	0.001-0.009
X	> 0.05 did not pass Benjamini-Hochberg correction. Importantly, the significant effects on the AUCi remained unaffected by Benjamini-Hochberg correction.

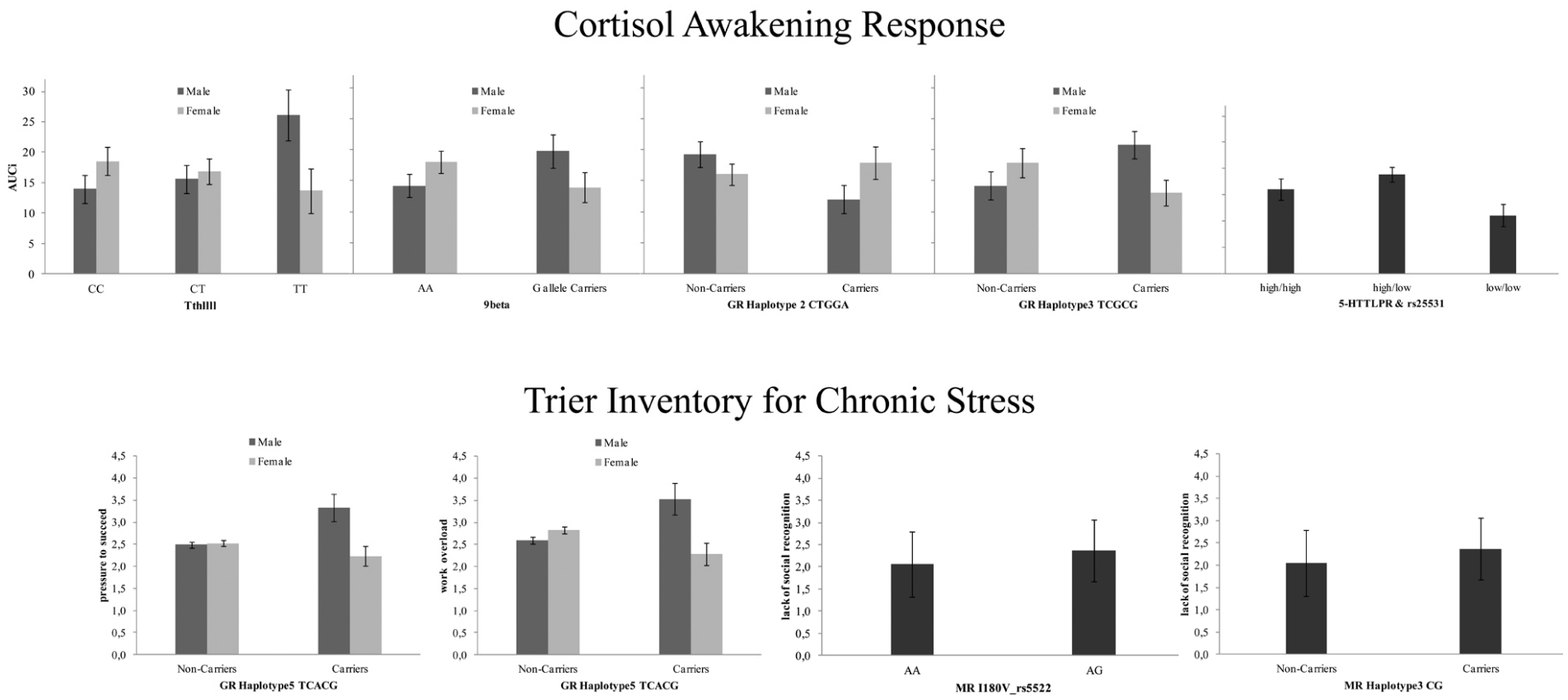

Figure 2.3: Mean values corresponding to significant ANOVA results

The upper section shows the mean area under the curve with respect to increase (AUCi) of the cortisol awakening response as a function of genotype or, respectively, haplotype. Separate light and dark grey columns represent male and female participants for genotypes and haplotypes that influenced AUCi in interaction with gender. Black bars represent the combination of male and female participants. The lower section shows mean ratings on scales of the Trier Inventory for Chronic Stress. Only means reflecting main effects or interaction effects that remain significant after the Benjamini-Hochberg correction are depicted. Error bars represent standard error of the mean.

2.5 Discussion

Genetic factors represent one of the components mediating individual variations in measurable physiological stress parameters and how these are reflected by emotional processes. Therefore, we investigated influences on stress regulation as indicated physiologically by baseline HPA axis activity and psychologically by measuring self-perceived stress in a healthy cohort. We examined the role of HPA axis-related genes (*GR*, *MR, 5-HTT*, and *BDNF*) with respect to the basal HPA axis activity represented by the CAR. We found *GR* to impact the CAR in a gender-dependent manner and *5-HTT* to impact the CAR independent of gender. No associations with *MR* or *BDNF* were observed. Whereas a recent study by Bolton et al. (2014) found an association of variants at the *SERPINA6/SERPINA1* locus with morning cortisol levels (but did not investigate the CAR), we identified that *GR* and *5-HTT* were associated with the dynamic change in cortisol levels following awakening.

It is an interesting finding that the SNPs studied here and being associated with the CAR affected AUCi rather than AUCg. AUCi is the change of the dynamics of cortisol, more related to the sensitivity of the system, emphasizing changes over time after awakening and has been the primary measure of the CAR. AUCg is the overall level of cortisol release, which depends on the baseline cortisol level present on waking. In general, AUCi has been considered to be a better indicator of sensitivity than AUCg (Pruessner et al., 2003). A basal HPA axis measure needs to be a sensitivity index because the function of this system is to react to challenges.

The GR, mediating the physiological reaction following acute stress as well as “normal” GC-mediated homeostasis, represents a key element in these processes. Different functional genetic variants of *GR* may lead to individual and gender differences in physiological and emotional response to stress. Correspondingly, we found gender-dependent associations of *GR* polymorphisms with the CAR (AUCi) as well as the TICS subscales “work overload”, “social overload”, “excessive demands from work”, “work discontent”, and “pressure to

succeed". All these associations are based on the two TICS blocks containing scales for excessive demands and deficits rather than the more general subscale chronic worrying.

Rosmond et al. first reported that men homozygous for the minor T allele of the *TthIII*I SNP showed higher basal cortisol secretion as estimated by total diurnal cortisol secretion (2000). Accordingly, we report here that the *TthIII*I SNP is associated with CAR (AUCi) in a gender-dependent manner, partly corresponding to Rosmond's study. Our data suggest that the *TthIII*I T allele is associated with a relatively higher GC sensitivity in HPA axis response at basal levels. *TthIII*I is localized in the promoter 1B region of *GR* (Cao-Lei et al., 2011) and may therefore be involved in the down-regulation of *GR* expression. Hence, *in vivo* studies showed that subjects homozygous for the T allele of *TthIII*I had significantly higher overall mean cortisol. In our study, men homozygous for the T allele of *TthIII*I also perceived lower "work discontent" according to TICS. Thus, it could be speculated that the higher baseline HPA axis activity is beneficial for the attitude towards work. Moreover, higher cortisol levels may prove advantageous for coping with work challenges.

Functional studies revealed a stabilizing effect of the 9beta polymorphism on GRbeta mRNA *in vitro*, leading to enhanced abundance of GRbeta protein (DeRijk et al., 2002). As a consequence, increased expression and stability of the GRbeta isoform leads to a relative GC resistance by an altered GRalpha to GRbeta ratio (Manenschijn et al., 2009). The higher CAR (AUCi) in males homozygous for the minor G allele found in this study could possibly be explained by this mechanism. Correspondingly, men carrying the minor C allele of NR3C1-1, being in high LD with 9beta, displayed a marginal association with a higher CAR (AUCi) and significant associations with higher "work overload" and "social overload". Also, men with haplotype 3, which harbours the minor alleles of *TthIII*I, NR3C1-I and 9beta showed higher AUCi. This is in consistence with the findings of the single SNPs.

Haplotypes may, in general, account more directly for genetic associations with different phenotypes compared to single SNPs due to combined effects of multiple sequence variants. Van Rossum et al. (Van Rossum et al., 2003) found an association of the *Bcl*I minor allele with GC hypersensitivity following dexamethasone treatment. In our study and in line with

previous findings, the *GR* haplotype 2 containing the *Bcl*I minor allele was associated with CAR in a gender-dependent manner. Regarding this result, the unequal distribution of the *GR* haplotype 2 between men and women is a coincidence that prohibits a true causal conclusion with regard to gene-gender interactions for that SNP in our study. However, the gender-dependency deserves further investigation. Male carriers of *GR* haplotype 5, which harbors the minor alleles of *Tthlll*I, NR3C1-1, E22E and 9beta, did not show any association with basal HPA levels but with TICS subscales "pressure to succeed" and "work overload".

In this study, polymorphisms of *GR* affected basal HPA axis activity in a gender-specific manner. This moderating influence corresponds to previous studies reporting gender differences in HPA axis response patterns (Kajantie & Phillips, 2006; Uhart et al., 2006). The interaction of *GR* genotypes and haplotypes with gender suggests that sex hormones might be involved in physiological and psychological stress regulation. Gender-specific differences of sex hormone composition and level might selectively affect the expression of certain genes (Weiss et al., 2005). Therefore, one specific functional polymorphism can result in different endophenotypes in males and females. Similar gender effects have been found for two *MR* SNPs modulating the suppression of the CAR after dexamethasone in a gender-specific manner, but did not associated with the CAR before dexamethasone treatment (Van Leeuwen et al., 2010). Moreover, the same group showed that carriers of *MR* haplotype 3 reported more chronic stress in terms of "excessive demands at work" and "social overload" compared to non-carriers (Van Leeuwen et al., 2011). In our sample and in line with the findings of previous studies, we showed an association of *MR* with TICS subscales but not with the CAR.

Accordingly, we found a significant association of *5*-HTTLPR with the CAR (AUCi), which might reflect the stimulation of serotonergic neurons, followed by the secretion of corticotropin-releasing hormone and subsequent release of cortisol (Heisler et al., 2007), Wüst et al. (2009) reported a gender-specific association with *5*-HTTLPR and basal secretion of cortisol, with males homozygous for the low active S allele showing the lowest CAR, whereas the CAR of females with the same genotype was highest. The results reported by

Wüst and associates were replicated in part by our study, as we found the AUCi level being lowest in the *5-HTT* low/low transcription activity group compared to high/high and high/low groups in both genders. With respect to self-perceived stress, subjects homozygous for the low/low allele of *5*-HTTLPR/rs525531 showed an association with higher "work discontent" - this may be related to the higher "neuroticism" personality trait described for this group in the initial study by Lesch and colleagues (Lesch et al., 1996). However, further studies are required to elucidate the role of serotonergic genes on basal cortisol levels with respect to gender.

The lack of any correlation of *BDNF* with the CAR or psychological traits is worth to be noted. In previous studies, the impact of *BDNF* on stress reactivity was investigated in clinical samples that reflect an imbalance of the stress system and especially the hormonal levels in itself (Pei et al., 2012). In our studies, we focused on healthy participants and the basal status without applying any stressor and therefore we did not find any association. Furthermore, BDNF signaling in the hippocampus may differently regulate the HPA axis activations in response to chronic or acute stressors.

More recently informed multilocus genetic profile analysis for biological relevant markers have been established (Bogdan, Hyde & Harri 2013). Sample size, minor allele frequencies of the polymorphisms and the methods of computing the scores influence the results of such an interesting attempt and restricted our analyses. We did not find any significant multilocus profile score for the HPA axis regulation. However, for future studies on the regulation of the HPA axis, it might be interesting to focus on low-frequency alleles in biological informative genes in larger samples in combination with those multilocus analyses.

Limitations

The sample size of the present study might constitute its major limitation, since the statistical power to detect very small effects of genetic associations could be considered low, although, given the sample size of N = 217, the statistical power to detect a small-to-medium-size effect of $\eta_p^2 = 0.04$ was $1 - \beta = 0.76$ for analyses comparing three genotype groups or,

respectively, 1 – β = 0.85 for analyses comparing two genotype or haplotype groups. Therefore, the significance of the reported associations and the complementary lack of associations of other SNPs or haplotypes have to be interpreted carefully. In addition, our strategy of extensive phenotyping, together with low allele frequencies of some of the variants investigated, inevitably leads to a relatively small size of subgroups. Since the heritabilities of the different TICS subscales vary between 5% and 45% (Federenko et al., 2006), detecting possible genetic influences for the scales with rather low heritabilities would require larger samples to find them. For future studies, it might be interesting to especially focus on examining low-frequency alleles in larger samples. Finally, the current sample consisted mainly of students; therefore, our results should not be generalized to other than a Caucasian college-aged population. As the TICS questionnaire is particularly designed to assess facets of chronic stress by occupational sources, *e.g.*, as to be expected in teaching professions (Van Leeuwen et al., 2011), the findings reported here have to be interpreted with caution.

2.6 Conclusion

We showed that compared to the other examined SNPs in a college-aged population only *GR* SNPs influenced both parameters of the basal HPA axis activity, the CAR and self-perceived stress. It is likely that *GR* variants can impact the efficacy of the GC signaling cascade and influence the downstream biology of the peripheral and central GC responsive system. AUCi as the best indicator of basal HPA reactivity and sensitivity seems to be affected more by genetic variation, whereas AUCg reflecting general hormonal levels might be affected rather by environmental factors such as methylation, which was not tested in our study. Additionally, we observed gender-dependent genetic effects on the CAR, which could contribute to the well-known gender differences in the vulnerability for stress-related diseases.

SM-Table 2.1: An overview of the previous studies on the common genetic variants in the human *GR*, *MR*, *5-HTT* and *BDNF* genes, and their correlation on HPA axis activity by different tests.

Genes	SNPs	rs number	Minor allele	MAF (%)	Testing HPA axis	Sample size/ cells	gender inter-action	Gene interaction	Ref.
GR	**T*thIII*I**	**rs1005295**	**T**	**±36**	Profile	284 men	N/A	TT homozygotes showed higher total and evening cortisol levels.	#1
	NR3C1-1	**rs10482605**	**C**	**±20**	DST	219	yes	C allele is associated with higher post DST cortisol in men, but with lower levels in women	#2
					TSST	219	yes	Male minor allele performed higher total cortisol	#2
					In vitro	brain cell lines	N/A	Minor allele showed reduced transcriptional activity	#3
	ER22/23EK	**rs6189/90**	**A**	**±4**	DST	490	no	No	#4
					TSST	206	no	No	#5
					In vitro	COS-1	N/A	Transcriptional activity in minor allele carriers is decreased	#6
	***Bcl*I**	**rs41423247**	**G**	**±33**	DST	216	no	GG carriers showed lower post dexamethasone cortisol	#7
					DST	206	yes	Male GG carriers showed the highest CAR peak after DST	#5
					TSST	206	yes	Male GG carriers displayed lowest saliva cortisol and lowest total cortisol	#5
	9beta	**rs6198**	**G**	**±19**	DST	206	yes	Male AG carriers showed the highest CAR peak after DST	#5
					TSST	206	yes	Male AG carriers showed the highest total cortisol response	#5
					GSST	553 adolescents	no	No	#8
					In vitro	COS-1		Minor allele increased the stability of hGRbeta mRNA	#9
MR	**MRI180V**	**rs5522**	**G**	**±11**	CAR	218	no	No	#10
					DST	218	yes	Male AA carriers showed higher CAR compared to male AG allele carriers	#10
					TSST	110	no	Minor allele show higher cortisol	#11

					In vitro	CV-1	N/A	MR180V allele revealed a mild loss of function using cortisol as a ligand	#11
	MR-2G/C	**rs2070951**	**C**	**±47**	Profile	563 older	no	C-allele carriers had lower cortisol levels than noncarriers	#12
					CAR	218	no	No	#10
					DST	218	yes	Female GG carriers show the largest CAR	#10
					GSST	553 adolescents	no	No	#8
					In vitro	COS-1	N/A	Minor allele less active *in vitro*	#10
5-HTT	**5-HTTLPR**	**rs 25531**	**S/G**	**±10**	CAR	216	yes	SS genotype showed the highest CAR in females, while showed the lowest CAR in males	#13
					DST	216	yes	No	#13
					TSST	216	yes	No	#13
					In vitro	lymphoblast cell line	N/A	The short variant polymorphism reduces transcriptional efficiency	#14
BDNF	**Val66Met**	**rs6265**	**A**	**±23**	TSST	100 men	no	Met allele carriers showed lower saliva cortisol level compare to subjects with the Val/Val genotype	#15
					In vitro	cultured neurons	N/A	Val/Met genotype impacted intracellular trafficking and activity-dependent secretion of BDNF	#16

Profile: cortisol daily profile; CAR: cortisol awakening response; DST: dexamethasone suppression test; TSST: Trier Social Stress; GSST: Groningen Social Stress Test; *In vitro*: functional test *in vitro* using cell lines; Ref.: references #1(Rosmond et al.,2000), #2(Kumsta et al.,2008), #3(Kumsta et al.,2009), #4(Van Rossum et al.,2006), #5(Kumsta et al., 2007), #6(Russcher H et al.,2005), #7(Stevens et al., 2004), #8(Bouma et al.,2010), #9(DeRijk et al., 2001), #10(van Leeuwen et al.,2010), #11(DeRijk et al., 2006), #12(Kuningas et al.,2007), #13(Wüst et al., 2009), #14(Lesch et al., 1996), #15(Alexander et al., 2010), #16(Egan et al.,2003)

SM-Table 2.2: Overview of the distribution for the different genotypes/haplotypes in dependence of gender, as well as information on the TICS scales and CAR measures (mean and standard deviation).

Gene	SNPs/Haplotype	rs number	Genotyping	Female (%)	Male (%)	Chi Square test parameter
GR	*Tth*III	rs1005295	CC	44 (0.20)	44 (0.20)	
			CT	53 (0.25)	45 (0.21)	χ2=0.732 df=2 P=0.694
			TT	17 (0.08)	12 (0.06)	
	NR3C1-1	rs10482605	TT	69 (0.32)	64 (0.30)	
			TC	40 (0.19)	34 (0.16)	χ2=0.345 df=2 P=0.842
			CC	5 (0.02)	3 (0.01)	
	E22E	rs6189	GG	104 (0.48)	97 (0.45)	χ2=1.721 df=1 P=0.190
			GA	11 (0.05)	5 (0.02)	
	R23K	rs6190	GG	99 (0.46)	95 (0.44)	
			GA	12 (0.06)	6 (0.03)	χ2=4.312 df=2 P=0.116
			AA	3 (0.01)	0	
	*Bcl*I	rs41423247	CC	58 (0.27)	35 (0.16)	
			GC	39 (0.18)	49 (0.23)	χ2=6.068 df=2 P=0.048
			GG	18 (0.08)	18 (0.08)	
	9beta	rs6198	AA	75 (0.35)	70 (0.32)	
			AG	35 (0.16)	29 (0.13)	χ2=0.458 df=2 P=0.795
			GG	5 (0.02)	3 (0.01)	
	Haplotype 1		CTGGCA	77 (0.35)	65 (0.30)	χ2=0.250 df=1 P=0.617
				38 (0.18)	37 (0.17)	
	Haplotype 2		CTGGGA	35 (0.16)	47 (0.22)	χ2=5.627 df=1 P=0.018
				80 (0.37)	55 (0.25)	
	Haplotype 3		TCGGCG	30 (0.14)	27 (0.12)	χ2=0.004 df=1 P=0.949
				85 (0.39)	75 (0.35)	
	Haplotype 4		TTGGGA	30 (0.14)	29 (0.13)	χ2=0.150 df=1 P=0.698
				85 (0.39)	73 (0.34)	
	Haplotype 5		TCAACG	10 (0.05)	5 (0.02)	χ2=1.209 df=1 P=0.272
				105 (0.48)	97 (0.45)	
	Haplotype 6		TTGGCA	7 (0.03)	3 (0.01)	χ2=1.217 df=1 P=0.270
				108 (0.50)	99 (0.46)	

MR	I180V	rs5522	AA AG	96 (0.44) 18 (0.08)	82 (0.38) 20 (0.09)	χ2=0.541 df=1 P=0.462
	-2C/G	rs2070951	CC GC GG	32 (0.15) 50 (0.23) 33 (0.15)	24 (0.11) 54 (0.25) 24 (0.11)	χ2=1.946 df=2 P=0.378
MR	Haplotype 1		GA	83 (0.38) 32 (0.15)	78 (0.36) 24 (0.11)	χ2=0.521 df=1 P=0.470
	Haplotype 2		CA	71 (0.33) 44 (0.20)	65 (0.30) 37 (0.17)	χ2=0.091 df=1 P=0.763
	Haplotype 3		CG	18 (0.08) 97 (0.45)	20 (0.09) 82 (0.38)	χ2=0.586 df=1 P=0.444
5-HTT	5-HTTLPR	rs25531	High/High High/Low Low/Low	27 (0.12) 60 (0.28) 28 (0.13)	27 (0.12) 51 (0.24) 24 (0.11)	χ2=0.260 df=2 P=0.878
BDNF	Val66Met	rs6265	AA AG GG	5 (0.02) 40 (0.18) 70 (0.32)	4 (0.02) 35 (0.16) 63 (0.29)	χ2=0.034 df=2 P=0.983

Trier inventory of chronic stress (TICS)	**All subjects**	**Female**	**Male**	**ANOVA**
work overload	2.71 ± 0.80	2.77 ± 0.83	2.63 ± 0.79	F=1.741 P=0.188
social overload	2.19 ± 0.89	2.29 ± 0.85	2.07 ± 0.92	F=3.345 P=0.069
excessive demands from work	1.96 ± 0.74	2.05 ± 0.76	1.86 ± 0.70	F=3.528 P=0.062
lack of social recognition	2.10 ± 0.74	2.11 ± 0.78	2.09 ± 0.71	F=0.037 P=0.847
work discontent	2.21 ± 0.92	2.17 ± 0.81	2.26 ± 1.02	F=0.469 P=0.494
social tensions	2.06 ± 0.74	2.12 ± 0.78	2.00 ± 0.70	F=1.358 P=0.245
pressure to succeed	2.51 ± 0.70	2.50 ± 0.74	2.53 ± 0.64	F=0.093 P=0.760
social isolation	2.26 ± 0.84	2.33 ± 0.86	2.19 ± 0.81	F=1.504 P=0.221
chronic stress worring	2.49 ± 0.85	2.70 ± 0.90	2.28 ± 0.74	F=13.671 P=0.00
cortisol awakening response (CAR)				
AUCg (nmol/l/h)	46.89 ± 19.18	46.50 ± 19.98	47.17 ± 19.51	F=0.066 P=0.798
AUCi (nmol/l/h)	16.44 ± 15.54	16.81 ± 16.52	16.01 ± 14.53	F=0.144 P=0.705

Abbreviations: AUCg: area under the curve with respect to ground, AUCi: area under the curve with respect to increase, BDNF: brain-derived neurotrophic factor, GR: glucocorticoid receptor, MR: mineralocorticoid receptor, 5-HTT serotonin transporter

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Chapter 3

The cardiovascular and hypothalamic-pituitary-adrenal axis response to stress is controlled by glucocorticoid receptor sequence variants and promoter methylation

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3.1 Abstract

Background: Gender, genetic makeup, and prior experience interact to determine physiological responses to an external perceived stressor. Here we investigated the contribution of both genetic variants and promoter methylation of the NR3C1 (glucocorticoid receptor) gene to the cardiovascular and hypothalamic-pituitary-adrenal (HPA) axis response to the socially evaluated cold pressor test (SeCPT).

Results: Two hundred thirty-two healthy participants were recruited and underwent the experiment. They were randomly assigned to either the SeCPT group (cold water) or a control group (warm water).The SeCPT group had a clear stress reaction; salivary cortisol levels, peak systolic and diastolic blood pressure all increased significantly compared to the control group. *GR* genotype (*TthIII*I, NR3C1-I, 1H, E22E, R23K, *Bcl*I and 9beta) and methylation data were obtained from 218 participants. Haplotypes were built from the *GR* genotypes, and haplotype 2 (minor allele of *Bcl*I) carriers had a higher cortisol response to the SeCPT in comparison to non-carriers (20.77 ± 13.22; 14.99 ± 8.42; p = 0.034), as well as independently of the experimental manipulation, higher baseline heart rate (72.44 ± 10.99; 68.74 ± 9.79; p = 0.022) and blood pressure (115.81 ± 10.47; 111.61 ± 10.74; p = 0.048). Average methylation levels throughout promoter 1F and 1H were low (2.76 % and 1.69 % respectively) but there was a strong correlation between individual CpGs and the distance separating them (Pearson's correlation r= 0.725, p= 3.03×10^{-26}). Higher promoter-wide methylation levels were associated with decreased baseline blood pressure, and when incorporated into a linear mixed effect model significantly predicted lower systolic and diastolic blood pressure evolution over time in response to the experimental manipulation. The underlying genotype significantly predicted methylation levels, particularly the homozygous *Bcl*I minor allele was associated with higher methylation in promoter 1H (p = 0.042).

Conclusion: This is one of the first studies linking epigenetic modifications of the *GR* promoter, receptor genotype and physiological measures of the stress response. At baseline, there were

clear genetic and epigenetic effects on blood pressure. The SeCPT induced a strong cardiovascular and HPA axis response and both systems were affected by the functional genetic variants, although methylation also predicted blood pressure reactivity. The return to baseline was predominantly influenced by the genomic sequence. Overall, the physiological response to the SeCPT is controlled by an exquisite mix of genetic and epigenetic factors.

3.2 Background

External challenges trigger hypothalamic-pituitary-adrenal (HPA) axis activation and cortisol secretion, maintaining homeostasis, and permitting adaptation (de Kloet, Joels, & Holsboer, 2005). Glucocorticoid receptor (GR, gene: *NR3C1*, OMIM +138040) protein isoforms and levels throughout all HPA axis tissues control glucocorticoid (GC) feedback, setting individual levels of stress reactivity and responsivity. A complex interplay of genetic and epigenetic mechanisms control GR levels, protein isoforms, and potentially the end phenotype. Twin research has suggested that part of the inter-individual differences in the stress response may be explained by genetic factors (De Geus, Kupper, Boomsma, & Snieder, 2007), and both rodent models and human studies show an environmental influence via epigenetic mechanisms (McGowan et al., 2009; Weaver et al., 2004; Witzmann et al., 2012). Both epigenetic and genetic factors influence the transcriptional control of the *GR* through the series of tissue-specific promoters found upstream of the 11 alternative *GR* first exons (Leenen et al., 2015; Turner & Muller, 2005; Turner et al., 2014).

It is well established that individual genetic variants of the glucocorticoid receptor affect both the basic cellular phenotypes i.e. *GR* expression levels (Cao-Lei et al., 2011), and the overall HPA axis stress response (reviewed in (Spijker & van Rossum, 2012)) through either an altered GC response or sensitivity. Numerous *GR* SNPs are in a high linkage disequilibrium resulting in commonly accepted haplotypes (Spijker & van Rossum, 2012) (SM-Table 3.1). Three haplotypes, *Bcl*I alone, *TthIII*I + *Bcl*I and N363S alone are all associated with increased sensitivity to GCs (van Rossum et al., 2003; van Rossum and Lamberts, 2004). The N363S polymorphism was associated with increased BMI, raised cholesterol levels and an increased risk for coronary artery disease (van Rossum & Lamberts, 2004). Inversely, two haplotypes *TthIII*I + 9β and *TthIII*I + 9β + ER22/23EK have been associated with GC resistance (Derijk et al., 2001). Importantly, there are a total of 12 known genetic variants throughout the 8 confirmed promoter regions controlling the expression of the 11 alternative first exons in the variable

5'untranslated region (UTR) of the *GR* (Breslin, Geng, & Vedeckis, 2001; Cao-Lei, et al., 2011). This 5'UTR is responsible for controlling tissue-specific alternative first exon expression, overall GR levels and isoforms (Cao-Lei et al., 2013; Turner et al., 2010; Turner & Muller, 2005; Turner, et al., 2014).

Epigenetic modifications such as DNA methylation, post translational chromatin remodelling and small RNA-based mechanisms have more recently been shown to contribute both independently or together with genetic variation to gene regulation. DNA methylation is unique, as it is the only epigenetic mechanism that may regulate gene expression, is clearly propagated through mitosis, whilst retaining its function (Zaidi et al., 2010). Although the associations of *GR* promoter methylation with diseases such as posttraumatic stress disorder (Yehuda et al., 2015), and depression (Alt et al., 2010; Klok et al., 2011; McGowan, et al., 2009; Oberlander et al., 2008) are well studied, there is very little evidence on how it influences HPA axis (re)activity, or any other aspect of the stress response such as cardiovascular reactivity. The available studies provide inconclusive data. High methylation levels were associated with an increased salivary cortisol response in infants (Oberlander, et al., 2008) and with a female-specific increased cortisol secretion after stress (Edelman et al., 2012). Conversely, increased methylation levels were also associated with a decreased response to pharmacological HPA axis stimulation (Tyrka, Price, Marsit, Walters, & Carpenter, 2012) or could be explained by differences in both education and lifestyle (de Rooij et al., 2012). The effect of DNA methylation on stress-related cardiovascular reactivity remains unexplored. These studies were limited to the proximal *GR* promoter regions thought to control tissue and stimuli specific GR levels (Turner, et al., 2010; Turner and Muller, 2005). The mechanisms underlying the effects of DNA methylation on gene expression are not, however, particularly well understood (Jones, 2012). The longstanding association of DNA methylation with gene silencing (reviewed in (Mazzio & Soliman, 2012)) does not reflect its functional complexity, orchestrating tissue-specific regulatory elements and expression patterns (Wan et al., 2015), marking alternative intra-genic promoters (Maunakea et

al., 2010), controlling alternative splicing (Jones, 2012; Laurent et al., 2010; Shukla et al., 2011), and even promoting gene transcription (Hellman & Chess, 2007; Irizarry et al., 2009; Wan, et al., 2015). The evidence currently available suggests that methylation in these regions of the *GR* do not only control the relative promoter activity, and levels of individual first exon transcripts, but also the final protein isoform and its cellular localisation (Turner, et al., 2010; Turner, et al., 2014).

Genetic and epigenetic factors work together to produce the overall response, reflected in the cortisol secretion and cardiovascular system activation to an external stressor, however, neither factor acts unilaterally. Whilst epigenetic factors, particularly DNA methylation, integrate the environment experienced with the genotype (Daskalakis & Yehuda, 2014), the underlying DNA sequence also has a large influence on methylation levels. In both genome-wide family based genetic studies and HapMap cell lines genetic variants affected DNA methylation without necessarily introducing new CpG methylation sites (Bell et al., 2011; Gertz et al., 2011). However, the only *GR* data available to our knowledge suggests that there is no association between the *Bcl*I variant and methylation of the GR promoter 1F in the human placenta, although none of the other promoters or exons were investigated. However, there was a potential association between methylation, genotype, and infant neurobehavioural outcomes (Bromer et al., 2013).

In this study, we investigated the relative contribution of genetic variants and promoter methylation of the *GR* to both cardiovascular and HPA axis stress reactivity. In a cohort of healthy adults, stress was induced using the socially evaluated cold pressor test (SeCPT), and cardiovascular reactivity was assessed from heart rate (HR), systolic and diastolic blood pressure (SBP / DBP) changes. HPA axis reactivity was assessed from salivary cortisol. We identified associations between promoter methylation and genetic variants, and analysed how these impact cardiovascular as well as HPA axis activity in response to the SeCPT.

3.3 Materials and Methods

Participants

Participants were recruited from the University of Trier (Germany) via e-mail and poster advertisements as previously reported (Larra, et al., 2014). Briefly, 232 healthy non- and low-frequency smokers (< 5 cigarettes per day) with a body mass index between 19 and 25 kg/m^2 were recruited, and 218 (115 women and 103 men) completed the experimental protocol. Subjects with an increased objective or subjective sensitivity to cold and any indication of circulatory disturbances or cardiovascular problems were excluded. All participants completed the validated German version of The Beck Depression Inventory (BDI-II), and donors with scores above 19, consistent with moderate depression, were excluded (Beck & Steer, 1984; Beck, Steer, & Brown, 1996; Hautzinger et al., 1994). As previously reported caffeinated and alcoholic drinks, physical exercise, and meals were not permitted in the 3 hours immediately preceding the experimental visit (Larra, et al., 2014). All experiments were performed between 1:30 and 6 pm. In accordance to the declaration of Helsinki, the research was approved by the ethical committee of the medical association of Rhineland-Palatinate, and all participants gave their written informed consent.

Socially evaluated cold pressor test

The socially evaluated cold pressor test (SeCPT) was performed as previously reported (Lass-Hennemann et al., 2011; Schwabe, et al., 2008). Briefly, participants assigned to the SeCPT group were asked to completely immerse their hand in either ice-cold (2-3 °C). Participants assigned to the control group were asked to completely immerse their hand in isothermic (35-37°C) water. Participants in the SeCPT group were under the social surveillance of an experimenter; their perceptions of social evaluation, uncertainty and lack of control were enhanced by warning them that the procedure may be painful, not communicating the duration of immersion during the test and informing them that their performance would be recorded for subsequent facial expression analysis. Participants assigned to the control group were not under

social surveillance and no video camera was present. All participants were asked to remove their hand from the water after 3 minutes. Immediately before and after cessation of the experiment participants were asked to make a subjective rating of arousal, stress, anxiety, tension, and activity on visual analogue scales ranging from 0 (“not at all”) to 100 (“very much”) in 10-point increments. Saliva samples were collected using absorbent cotton rolls (Salivette, Sarstedt, Nuembrecht, Germany). Samples were stored at -20 °C until analysis. Salivary cortisol was measured in duplicate using a time-resolved fluorescence immunoassay (Dressendörfer et al., 1992). As previously reported, intra-assay and inter-assay coefficients of variance were 4.0-6.7 % and 7.1-9.0 %, respectively (Macedo et al., 2008). Heart rate (HR) and blood pressure (SBP, DBP) were measured throughout the experiment using the Dinamap System (Critikon; Tampa, Florida, USA) with the cuff placed on the right upper arm.

Genetic analysis

DNA isolation

DNA was extracted from EDTA anti-coagulated blood using the salting out protocol of Miller et al. (Miller, Dykes, & Polesky, 1988). Genomic DNA concentration was measured on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). DNA was stored at -20 °C prior to bisulfite modification and pyrosequencing or genotyping.

Methylation analysis

Bisulfite modification and pyrosequencing were performed as previously reported (Alt, et al., 2010; Cao-Lei, et al., 2013; Turner et al., 2008). Briefly, 400-ng genomic DNA was bisulphite converted using the EpiTect-Bisulfite Kit (Qiagen) according to the manufacturer’s protocol. Promoters 1F and 1H were subsequently amplified by PCR and quantitatively pyrosequenced as previously reported (Alt, et al., 2010; Cao-Lei, et al., 2013). Pyrosequencing was performed using a PyroMark ID system, and methylation levels of each CpG dinucleotide was analyzed using the Pyro Q-CpG software (version 1.0.9, Biotage). Positive controls were generated by

incubation of genomic DNA with SssI, and bisulphite conversion efficiency was calculated from the conversion rate of cytosine to thymidine when not immediately followed by a guanidine as previously described (Alt, et al., 2010; Cao-Lei, et al., 2013).

Genotyping and Haplotype Construction

The *GR* polymorphisms *TthIII*I (rs10052957), NR3C1-I (rs10482605), the promoter 1H SNP (rs10482614), ER22/23EK (rs6189 and rs6190), *Bcl*I (rs41423247) and 9beta (rs6198) were genotyped using a single nucleotide primer extension reaction, for which specific primers for each SNP were used in the SNP Start Master Mix kit from Beckman Coulter and where fragments were analyzed with the CEQ8000 Genetic Analysis System (Beckman Coulter, Inc, Germany). Detailed information about primer sequences, PCR conditions and purification methods are available in the supplementary information (SM-Table 3.2). Sanger sequencing of promoter 1H was performed as previously described (Cao-Lei, et al., 2011). All SNPs were tested for Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) was assessed for all 7 SNPs using Haploview 4.2 (Barrett, Fry, Maller, & Daly, 2005), and LD scores were expressed as D'. Individual haplotypes were reconstructed using PHASE, version 2.1 (Stephens & Donnelly, 2003; Stephens, Smith, & Donnelly, 2001) (http://stephenslab.uchicago.edu/software.html#phase), which uses an algorithm based on coalescence-based Bayesian Haplotype inference for predicting haplotypes from genotype data, combining modelling strategy with computational strategies.

Data reduction and Statistical Analysis

GR genotyping data was reduced by dichotymizing the SNPs with low minor allele frequencies combining the hetero-and homozygous carriers of the minor allele in one group. Cardiovascular data (HR, SBP, DBP) was reduced by extracting the mean increase of dependent variables from baseline to the peak after the water task and mean decrease from the peak to the recovery period. These are referred to as e.g: "SBP peak", "SBP increase" and "SBP decrease". Baseline

was considered as an average value of three measurements before water task. Recovery period was calculated using the three measurements after the water task. Cortisol data was reduced to the area under the curve with respect to ground (AUCg) and increase (AUCi; (Pruessner et al., 2003)). All variables were tested for normality graphically using kernel density plots and Normal Q-Q plots and numerically using the Shapiro and Kolmogorov-Smirnov tests and the values of kurtosis and skewedness from the corresponding functions of the R package “moments”. Principal component (PCA) and internal consistency analysis (Cronbach’s alpha) were performed on all questionnaire derived data. Spherical representations of a correlation matrix and variance inflation factors (VIF) were used to identify correlations and co-linearity between covariates and explanatory variables. Variables with VIF > 5 are considered co-linear and excluded from all subsequent models and analyses. Confounding factors were evaluated in a bivariate analysis for association with methylation status and test group using linear regression and Pearson’s or Spearman’s correlations or the chi-squared test, respectively. Any variable showing a significant association ($p < 0.05$) was included in a linear mixed effect model as a covariate. Linear mixed effects model selection was based on maximum likelihood, and an autoregressive matrix was chosen for the residuals covariance structure. All the analyses were performed using R, version 3.0.1 (The R foundation for Statistical Computing) except genotype and haplotype analyses for which general linear models (GLMs) were computed using SPSS 20.0 to assess the between-subjects effect genotype as well as the interaction time x genotype x groups for the cortisol level. Differences were considered to be significant when $p < 0.05$ after suitable post hoc correction in all statistical analyses. The Bonforroni correction was used for all bivariate analyses, and Tukey's HSD was used for the repeated measures ANOVA (from R package Tukey HSD).

3.4 Results

Study population and randomisation

A homogenous cohort of 232 undergraduate students with minimal lifestyle differences were recruited, and data from 218 (103 males and 115 females) were analysed (Figure 3.1).

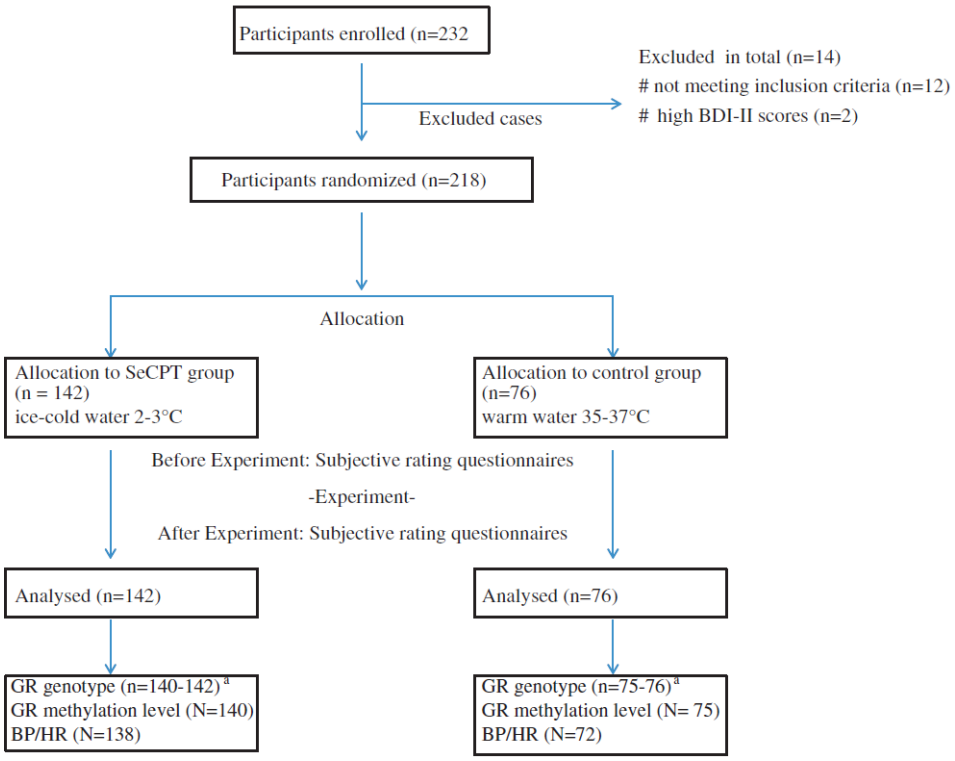


Figure 3.1 Recruitment summary for all donors contacted, participating, exiting and analysed after completion of the study

Two participants had BDI-II scores of 25 and 26, indicative of moderate depression and were excluded from all subsequent analyses. The mean BDI-II score was 5.976, within the minimal range (0-13). Although the mean BDI-II score for female participants was slightly higher (p = 0.011) compare to males, no difference has been found between the SeCPT and control group (p = 0.438). When included in the study, participants were given a date for the experimental session. Upon arrival on that date, they were assigned to either the SeCPT or control group in a 2:1 alternating order. At baseline, there were no group differences in any of the variables tested (Table 3.1).

Table3.1: Summary of participant characteristics and group gender repartition

	seCPT group	Control group	Full population	*p* value[a]
Sex (male)	70	35	105	0.736
Age	22.89 ± 2.7	23.27 ± 3.0	23.01 ± 2.8	0.356
BMI	22.1 ± 2.3	22.5 ± 2.4	22.2 ± 2.3	0.243
BDI	6.1 ± 5.3	5.5 ± 4.5	6.0 ± 5.1	0.438
Arousal (0–100)	20.1 ± 19.3	23.2 ± 20.1	23.8 ± 19.5	0.769
Stress (0–100)	25.2 ± 21.8	22.4 ± 21.3	24.3 ± 21.6	0.379
Anxiety (0–100)	12.1 ± 15.6	7.6 ± 9.5	10.6 ± 14.1	0.010
Tension (0–100)	17.8 ± 4.8	17.6 ± 5.2	17.7 ± 4.9	0.803
Activity (0–100)	26.3 ± 6.5	27.6 ± 6.1	26.7 ± 6.4	0.153
	Males	Females	Analysed population	
Complete cohort	103 (47.2 %)	115 (52.8 %)	218 (100 %)	
seCPT	70 (66.66 %)	77 (68.14 %)	147 (66.97 %)	
Control	35 (33.33 %)	36 (31.86 %)	72 (33.03 %)	

[a]comparsion of SeCPT vs Control group

SeCPT induced a physiological and subjective psychological stress response

As previously reported (Larra et al., 2014), salivary cortisol levels were significantly increased in the SeCPT group compared to the control group (p_{AUCg} = 0.001; p_{AUCi} < 0.001, t-test). Peak SBP and DBP levels were significantly increased from baseline in the SeCPT group compared to the control group. However, both SBP and DBP were comparable between the SeCPT and control groups in both the baseline and recovery periods (p = 0.299 and p = 0.712, ANOVA between the SeCPT and the control group). Bivariate analysis by sex showed a significantly greater increase in SBP levels in men (p = 7,02 x 10^{-8}), and a trend towards a greater increase from baseline to peak levels (p = 0.093). There was no effect of sex on diastolic blood pressure or heart rate increase, decrease, or peak levels (p > 0.1). Participants rated the SeCPT significantly more stressful, and had a significantly higher levels of arousal, anxiety, activity, and tension (all p < 0.01 paired t-tests) compared to the control group. Subjective ratings were not dependent on gender (p > 0.05), although female participants tended towards increased anxiety in the SeCPT group compared to control (p = 0.057).

Genotype and haplotype analysis in the cohort

GR genotyping was completed for *Tth*III, NR3C1-I, 1H, E22E, R23K, *Bcl*I and 9beta in 217, 215, 218, 218, 216, 218, and 218 participants, respectively. Due to their low frequencies, both hetero-and homozygous carriers were combined into one group for R23K (GA = 18, AA = 3) and promoter 1H (GA = 55, AA = 3). Minor allele frequencies (Table 3.2), and LD scores (D') (Figure 3.2) were in line with previously reported data (Kumsta et al., 2009). The haplotype structure was successfully created with PHASE for all the available data points, and those with frequencies above 5% are illustrated in Figure 3.2. Haplotype 1 (C-T-G-G-G-C-A) had a frequency of 61.9% and consisted of the major alleles of each SNP. Haplotype 2 (C-T-G-G-G-G-A) with 36.8% contained the minor alleles of *Bcl*1. Haplotype 3 (T-C-G-G-G-C-G) included the minor allele of T*thIII*1, NR3C1-I and 9beta and showed a frequency of 26.4%. Haplotype 4 (T-T-A-G-G-G-A) with 19.5% contained the minor allele of T*thIII*1, 1H

and *Bcl*1. Haplotype 5 (T-C-G-A-A-C-G) with 6.5% contained the minor allele of T*thIII*1, NR3C1-I, *ER22E*, *R23K* and 9beta. Haplotype 6 (T-T-G-G-G-G-A) included the minor allele of T*thIII*1 and *Bcl*1 showed a frequency of 5.2%. The haplotype structure was similar to that previously reported (Cao-Lei, et al., 2011; Kumsta, et al., 2009).

Table 3.2: Descriptive data of *NR3C1* single markers in 218 participants using Haploview

Marker	Position	obsHET	predHET	HWpval	% geno	MAF	Alleles
*TthIII*1	−142766894	0.463	0.458	1.0	99.6	0.355	C:T
NR3C1-I	−142763714	0.356	0.331	0.368	98.7	0.209	T:C
1H	−142762357	0.241	0.232	0.8032	100.0	0.134	G:A
E22E	−142760532	0.075	0.072	1.0	100.0	0.037	G:A
R23K	−142760530	0.084	0.105	0.0437	99.1	0.055	G:A
*Bcl*1	−142758768	0.408	0.462	0.0988	100.0	0.362	C:G
9beta	−142637814	0.307	0.306	1.0	100.0	0.189	A:G

HWpval: p value for Hardy-Weinberg-Equilibrium; MAF: minor allele frequency; obsHET: observed heterozygosity; position: chromosomal location; predHET: predicted heterozygosity; % Geno: Genotyping frequency

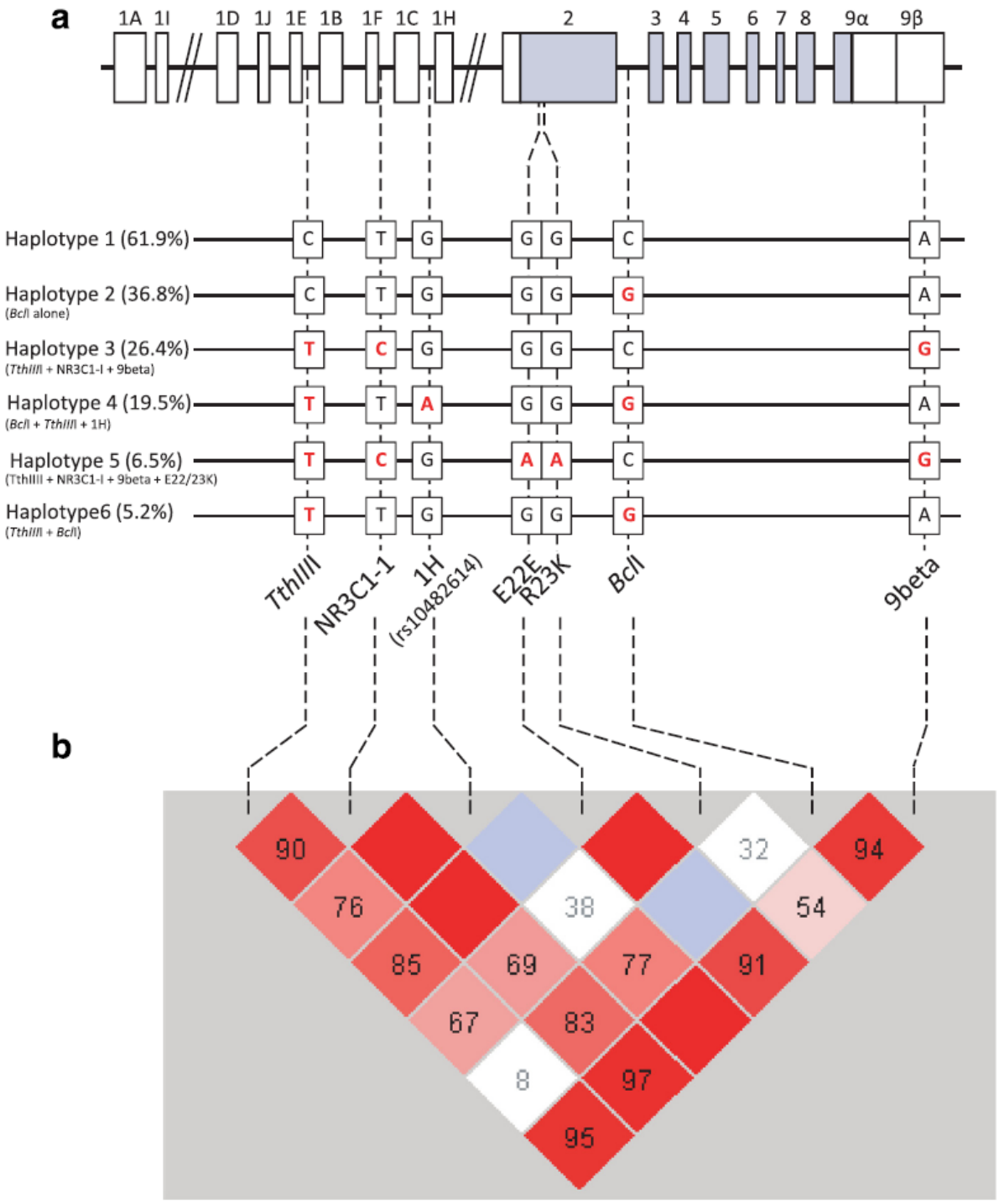


Figure 3.2: The genomic organisation, sequence variants and haplotype structure of the glucocorticoid receptor gene (*NR3C1*). **a** A schematic representation of the *NR3C1* genomic organisation. Rectangles represent transcribed exons. Exons 1A–1I are alternatively spliced to a common acceptor site at the start of exon 2. White exons are non-coding, grey exons represent the coding sequence. The lower section of the panel shows the six haplotypes observed, their constituent variants and frequencies. Minor alleles are represented by bold red letters. **b** The linkage disequilibrium (LD) structure of the *NR3C1*. LD between two variants are given by color, *blue/grey* no LD; *white*, limited LD; *light red* to *dark red*, medium to strong LD. Numbers within the LD *diamonds* represent the value of D prime (D') between the two loci. D' is statistic normalised parameter of disequilibrium

Haplotype associations with HPA axis reactivity

AUCg and AUCi were used as the dependent variable in separate between-participants ANOVAs with the factors SeCPT group and genotype (with each SNP and each haplotype as the genotype factor). AUCg was influenced by a significant interaction of haplotype 2 (*Bcl*I alone) and SeCPT group (20.77 ± 13.22; 14.99 ± 8.42; $p = 0.034$). Scrutinizing the structure of this interaction effect, effects analyses showed that carriers of the haplotype 2 had a significantly higher AUCg than non-carriers in the SeCPT group (20.77 ± 13.22; 14.99 ± 8.42; $p = 0.003$), whereas carriers and non-carriers did not differ significantly in the control group (SM-Table 3.3).

Association of haplotypes with HR and SBP

To evaluate the influences of genotypes and haplotypes on SBP and HR, a series of between-participants ANOVAs were performed. The results are summarized in SM-Table 3.3. There were significant main effects on the baseline, peak and recovery with higher HR for carriers of the haplotype 2 (72.44 ± 10.99; $p = 0.022$, 74.05 ± 12.25; $p = 0.023$, 69.50 ± 9.48; $p = 0.027$, respectively) as compared to non-carriers (68.74 ± 9.79; 70.65 ± 11.74; 66.20 ± 9.28). We observed, independent of the experimental group, a significantly higher decrease of HR after the water task in carriers of haplotype 3 compared to non-carriers (6.93 ± 9.80; 3.55 ± 7.03; $p = 0.016$).

Homozygote carriers of the minor G allele of the SNP *Bcl*I showed higher baseline HR (72.51 ± 11.57; 68.39 ± 9.13; $p = 0.048$). “Bpm increase” was influenced by a significant interaction of *Bcl*I and SeCPT group (-0.76 ± 7.89; 4.61 ± 9.02; $p = 0.029$). Simple effects analyses showed that homozygous carriers of the C allele had a significantly higher increase than homozygous carriers of the G allele in the SeCPT group (-0.76 ± 7.89; 4.61 ± 9.02; $p = 0.006$), whereas CC and GG carriers did not differ significantly in the control group (SM-Table 3.3). In our study, the G allele of *Bcl*I is a risk allele for higher HR. Carriers did not

respond to a stressor in the same way as the carriers of the C allele, that is, with an increase in HR (Figure 3.3).

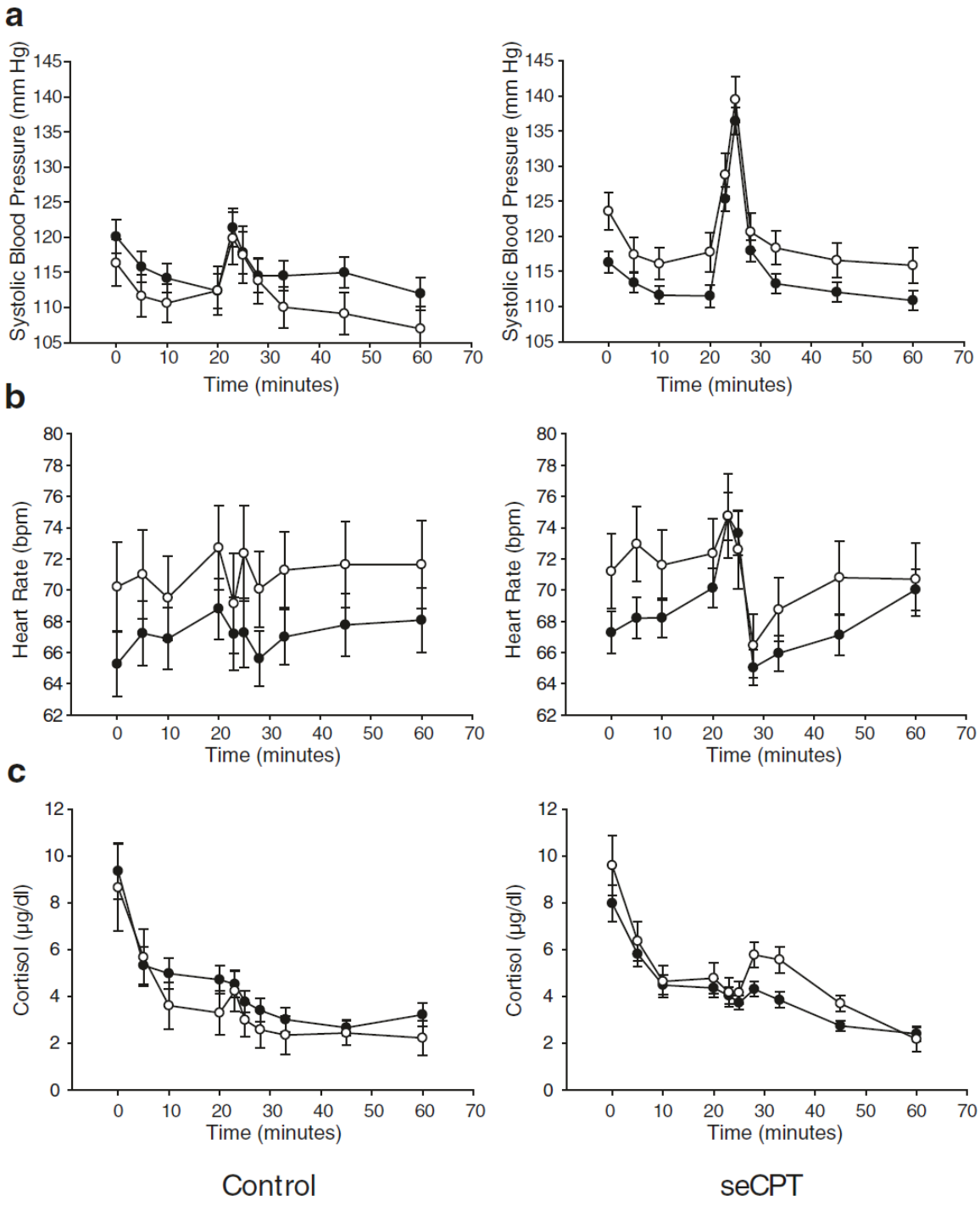


Figure 3.3 Haplotype 2 (*Bcl*I alone) effects of the warm (control) or ice-cold water (SeCPT) condition on SBP, heart rate and cortisol over the course of the experiment. **a** Systolic blood pressure in millimeter of mercury after the control (*left panel*) or cold water (*right panel*). **b** Heart rate in beats per minute after the control (*left panel*) or cold water (*right panel*). **c** Salivary cortisol levels in the control (*left panel*) or cold water (*right panel*). The SeCPT or warm water was administered at 23 min and lasted 3 min. In all panels, *filled circles* are homozygous wild-type (CC) participants and *open circles* are homozygous minor allele (GG) participants. Data are the mean ± the standard error of the mean.

There was an interaction between haplotype 2 and group regarding SBP baseline (115.81 ± 10.47; 111.61 ± 10.74; p = 0.048). Only in the SeCPT group, carriers of the haplotype 2 had a significantly higher “SBP baseline” than non-carriers (115.81 ± 10.47; 111.61 ± 10.74; p = 0.025). This effect of haplotype may have emerged only in the SeCPT group because of the higher participant number in there. There was also an interaction between haplotype 3 and group regarding the recovery SBP (112.54 ± 11.22; 116.41 ± 10.68; p = 0.019). Non-carriers in the SeCPT group had higher recovery SBP than the non-carriers in the control group (116.41 ± 10.67; 112.75 ± 11.88; p = 0.049), whereas carriers in both groups did not differ.

GR promoter methylation levels and distribution in the cohort

Methylation analysis was performed on 218 participants. In total, 14 participants (6%) were excluded, 3 due to missing methylation data, 10 for no HR/SBP/DBP data, and 1 for which both data were missing. Average methylation levels of individual CpGs in promoters 1F and 1H were 2.76 % and 1.69 %, respectively, and were directly comparable to previous reports from human white blood cells. Methylation levels did not exceed 14% for any donor at any position throughout promoters 1F and 1H. As reported for previous cohorts (Cao-Lei, et al., 2013), methylation levels of individual CpGs in close proximity strongly correlated in both promoter 1F and 1H (Pearson’s correlation r = 0.725, $p = 3.03 \times 10^{-26}$; Figure 3.4), confirming CpG methylation levels were co-regulated over short distances, probably in small clusters.

As methylation levels correlated in clusters, promoter-wide sum methylation levels were investigated. Promoter 1H sum methylation levels were significantly higher in women than men (Mann Whitney Rank Sum Test, $p < 0.01$; Figure 3.4), although there was no difference for promoter 1F sum methylation levels (Mann-Whitney rank sum test, p = 0.91; Figure 3.4). This difference was maintained for methylation summed throughout the two promoters (p=0.038, Mann-Whitney rank sum test). Although sum methylation levels for promoters 1F and 1H were not normally distributed ($p < 0.001$ Shapiro and Kolmogorov-Smirnov tests)

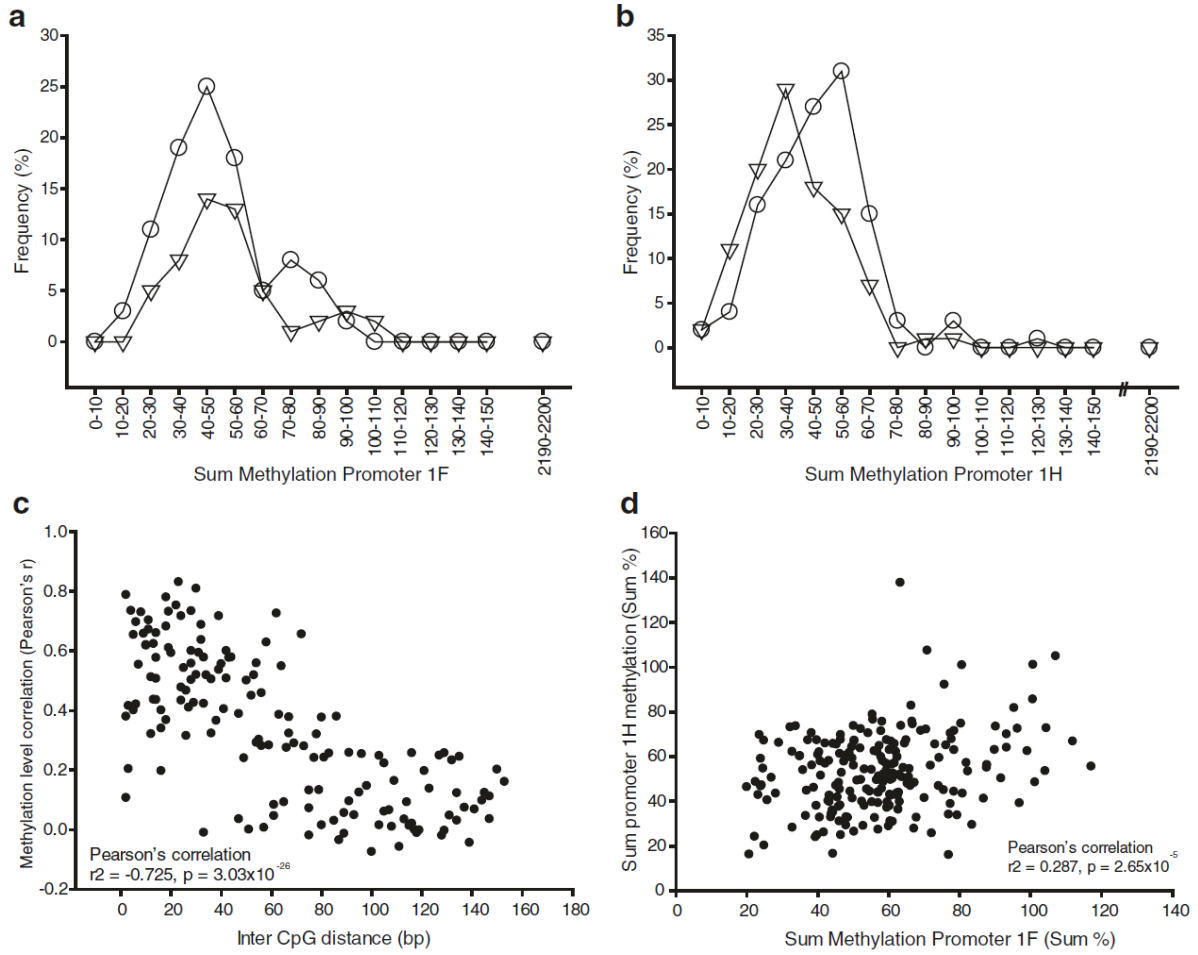


Figure 3.4 Methylation of the NR3C1 promoters 1F and 1H. **a** Frequency distribution of the sum of the methylation throughout promoter 1F. Female donors, *open circles*; male donors, *open triangles*. **b** Frequency distribution of the sum of the methylation throughout promoter 1H. Female donors, *open circles*; male donors, *open triangles*. **c** Pearson's correlation coefficients were calculated for all CpG pairs and subsequently plotted against the physical distance measured in nucleotides, demonstrating that the closer two CpG nucleotides are, the stronger their correlation in methylation levels. Each data point represents Pearson's correlation coefficient for one pair of CpGs from all donors. **d** Pearson's correlation in methylation levels between sum methylation levels in promoter 1F and 1H. Each data point represents one participant

there was a weak but significant Pearson's correlation between the two promoters (r = 0.287, $p = 0.65 \times 10^{-4}$. Figure 3.4), suggesting the clusters may also cover complete promoters.

For our linear mixed effects model, methylation levels, despite the potential loss of statistical power, were treated as a binary variable after a median split. After median split, the difference in methylation between the sexes were reflected in the ~60% : 40% ratio of males to females in the low methylation group and the inverse in the high methylation group. There was no bias in their randomisation into the SeCPT or control group (Table 3.3).

Table 3.3: Gender repartition after median split on methylation level

	Males, *n* (%)	Females, *n* (%)	Full population
Low methylation	60 (56.07)	47 (43.93)	107 (49.08)
seCPT group	42	31	73
Control group	18	16	34
High methylation	44 (39.64)	67 (60.36)	111 (50.92)
seCPT group	27	31	73
Control group	17	21	38

Methylation level predicts SBP and DBP

To evaluate the link between methylation of the two *GR* promoters studied and the stress response, a series of bivariate analyses, correlation tests and a focused principal component analysis were performed, identifying the factors that were subsequently used in a linear mixed effects model of the stress response (Table 3.4). SBP was identified as the variable to be explained, and *test group, methylation group, sex, arousal after the SeCPT, arousal change, tension, discomfort, stress after the SeCPT,* and *stress change* were retained as explanatory variables for further analysis. A maximum likelihood linear mixed effects model with an autoregressive matrix for the covariance structure of the residuals was constructed. Model residuals were normally distributed and centred on zero, suggesting a valid statistical model. This model confirmed the link between methylation levels and SBP, as well as having a significant effect on SBP evolution over time (Table 3.4).

Table 3.4: Linear mixed effects models for systolic and diastolic blood pressure

	Value	SEM	DF	*t* value	*p* value
SBP					
Intercept	114.154	1.701	1864	67.115	0
High methylation group	−2.362	1.344	199	−1.757	0.0083
Time	−0.375	0.099	1864	−3.758	0.0002
Arousal	−0.052	0.037	199	−1.395	0.0761
Stress	0.028	0.035	199	0.805	0.3964
Test group (seCPT vs control)	2.313	1.652	199	1.400	0.2042
DBP					
Intercept	67.572	1.067	1864	63.350	0
High methylation group	−2.225	0.942	200	−2.361	0.0192
Time	−0.085	0.068	1864	−1.254	0.2101
Arousal	−0.03184	0.026	200	−1.203	0.2302
Stress	0.025	0.025	200	1.004	0.3164
Test group (seCPT vs control)	2.551	1.174	200	2.173	0.031

The interactions between SeCPT group x time and methylation level x time were assessed, but not significant ($p > 0.05$). A second mixed effects model was generated for DBP (Table 3.4). This model gave a similar distribution of the residuals, and was equally valid. DBP was significantly associated with the methylation grouping, and SeCPT group ($p = 0.019$ and 0.031, respectively), although *time, arousal,* and *stress* were not associated ($p > 0.1$). The effect of methylation group on SBP is illustrated in Figure 3.5.

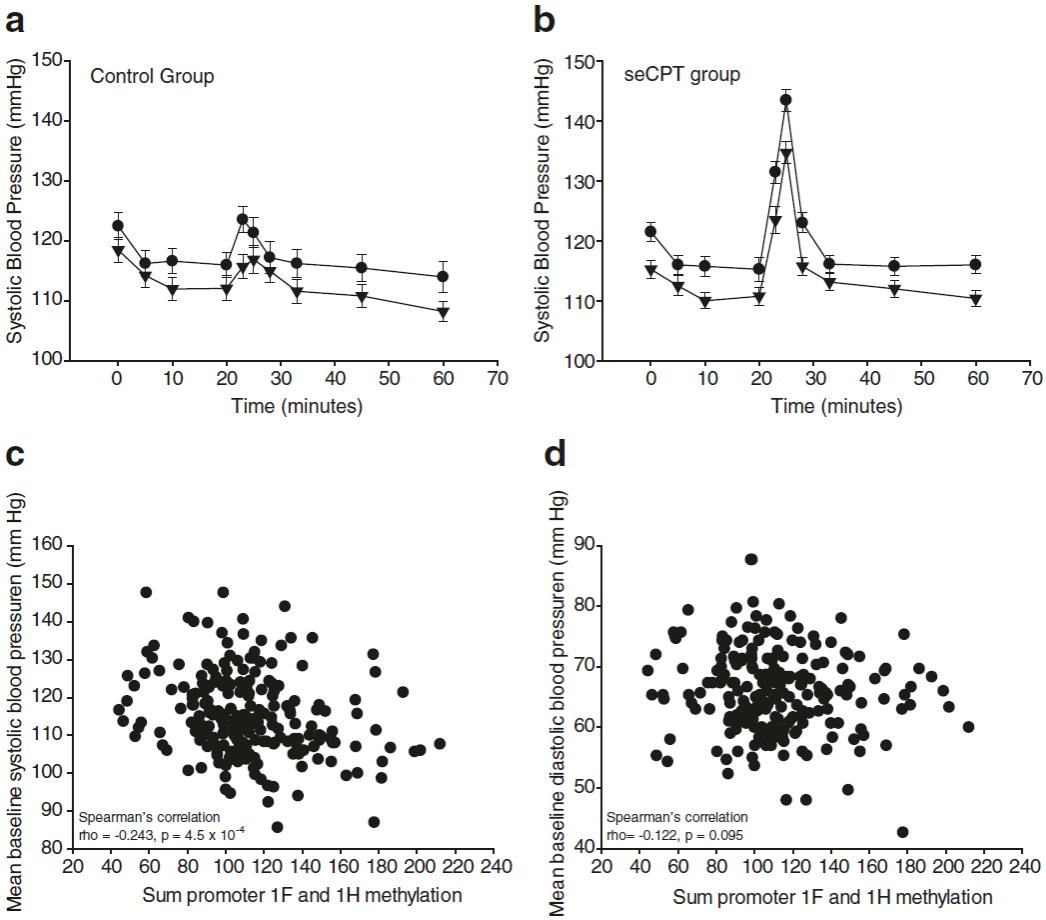


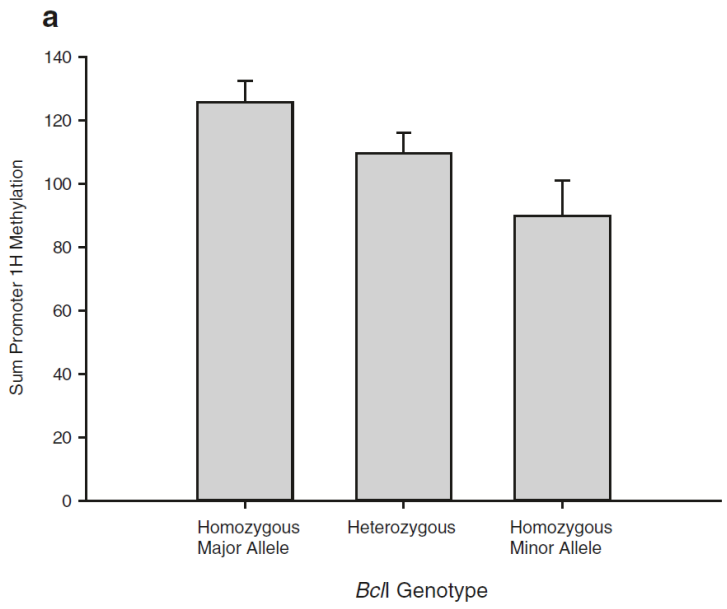
Figure 3.5 *NR3C1* promoter methylation effects on the systolic blood pressure response to the warm (control) or ice-cold water (SeCPT) over the course of the experiment. Donors were split by median sum methylation levels. Systolic blood pressure in millimeter of mercury after the control (**a**) or cold water (**b**) was administered at 23 min and lasted 3 min. In both panels: *filled circles*, low methylation group; *filled triangles*, high methylation group. Data are the mean ± standard deviation. **c** Correlation between the mean baseline SBP and sum promoter 1F and 1H methylation levels. All participants are included, and each data point represents one participant. **d** Correlation between the mean baseline DBP and sum promoter 1F and 1H methylation levels. All participants are included, and each data point represents one participant. Baseline SBP and DBP mean of the three time-points immediately preceding the warm or cold water exposure

In the statistical model for both SBP and DBP, methylation data remained a valid predictor, despite the loss of power after dichotomisation. As a separate confirmation that the sum

methylation of *GR* promoters 1F and 1H was significantly associated with peak SBP levels, methylation data was analysed as a continuous variable. Spearman's correlations were performed, confirming this link (rho = -0.243, p = 0.00045; Figure 3.5). However, DBP only had a trend towards associating with methylation (rho= -0.122, p = 0.095; Figure 3.5).

Association of haplotype and methylation levels

Linear association tests revealed a link between the *Bcl*I-minor allele and promoter 1H methylation (p = 0.00417 Figure 3.6) although this was not significant for promoter 1F methylation. This link was confirmed using the chi-squared test on the median split methylation group, where the homozygous minor allele carriers were associated with promoter 1H methylation (p = 0.0423).



b

*Bcl*I	Promoter 1H Methylation	p value
Homozygous Major Allele		$<2 \times 10^{-16}$
Heterozygous	-13%	0.0899
Homozygous Minor Allele	-29%	0.0042

Figure 3.6 Statistical interpretation of the link between haplotype 2 (*Bcl*I alone) and promoter methylation. **a** Mean methylation level of donors separated by *Bcl*I genotype. **b** Summary linear association test results

As the *Bcl*I genotype is part of haplotype 4 (*Bcl*I + *TthIII*I + 1H), the association between methylation and haplotype 4 was analysed. Haplotype 4 tended to associate with both promoter 1F and 1H methylation levels (linear association, $p = 0.067$ and 0.066) although the combined methylation grouping was not linked to the haplotype ($p = 0.102$, chi-squared test). Similarly, haplotype 5 (*TthIII*I + NR3C1-I + 9beta + ER22/23EK) showed an association trend to promoter 1F methylation levels ($p = 0.064$, linear association), but did not associate with promoter 1H methylation levels ($p = 0.921$, linear association).

As the *Bcl*I genotype has been previously reported to be in LD with variants in promoter 1H (rs10482614) (Cao-Lei, et al., 2011), Sanger sequencing of this promoter was performed. The minor alleles of rs10482614 and rs41423247 (*Bcl*I) were observant at frequencies of 29.3% and 36.2%, respectively. As previously reported, rs10482614 was in LD with *Bcl*I (Cramer's association coefficient, $V = 0.324$, p value = 3.032e-08, $r^2 = 0.16$ and $d' = 0.77$; Figure 3.2). Although the presence of the minor allele of rs10482614 (G/A) removes a CpG dinucleotide, there was no significant link between the presence of the rs10482614 minor allele and methylation of the 1H promoter ($p = 0.316$)

3.5 Discussion

The individual response to an external stressor is dependent on a panoply of factors. Here, we report the impact of *GR* promoter DNA methylation and sequence variants on the physiological response to stress. Increased *GR* 1F and 1H methylation levels were significantly associated with decreased baseline blood pressure. *GR* haplotype 2 (minor allele of *Bcl*I) carriers had a higher cortisol response to the SeCPT. In addition, *GR* haplotype 2 carriers had higher heart rate and higher blood pressure independent of experimental group. Haplotype 3 carriers had a stronger heart rate decrease post stress. A major novel finding was that the *GR Bcl*I minor allele was associated with higher *GR* promoter 1H methylation.

The physiological response to stress involves the sympathetic nervous system (SNS) and the HPA axis. The cold pressor test (CPT), introduced by Hines and Brown, reliably

increases blood pressure, a thermoregulatory reflex as well as a global activation of the sympathetic nervous system under standardized conditions (Roy-Gagnon et al., 2008). Physiological responses, including vasoconstriction, increased skin conductance (Buchanan, Tranel, & Adolphs, 2006) and elevated blood pressure (Lovallo, 1975; Peckerman et al., 1994) are induced. The addition of a social evaluative component in the SeCPT adds a substantial HPA axis activation. However, rapid elevations in blood pressure trigger baroreflex mechanisms counteracting the heart rate increase. Consequently, blood pressure is considered the appropriate measure of cardiovascular reactivity in the SeCPT (Schwabe, Haddad, & Schächinger, 2008), although we observed differences is both blood pressure and heart rate. The well-defined timing of the SeCPT allowed us to successfully examine the baseline, immediate post-stress period and the return to baseline. At baseline, there were clear genetic and epigenetic effects on blood pressure. The SeCPT induced a strong SNS and HPA axis response and both systems were affected principally by genomic variants. The return to baseline was predominantly influenced by the genomic sequence

Genomic variants had a significant effect on cardiovascular parameters. *GR* haplotype 2 (minor allele of *Bcl*I) carriers had higher baseline, peak and recovery period heart rate, and haplotype 3 carriers (minor allele of *TthIII*I, NR3C1-I and 9beta) had a stronger heart rate decrease post stress, both independent of the experimental group. Both of these haplotypes have previously been explored in detail, corresponding to haplotypes 4 and 2 from Cao-Lei et al (Cao-Lei, et al., 2011) and Kumsta *et al.* (Kumsta, et al., 2009). Haplotype 2 appears to play a central role in determining the cardiovascular stress response. However, *Bcl*I is an intronic polymorphism, 646 bp downstream of the common exon 2 that has generally been found to associate with increased GC sensitivity (Manenschijn, van den Akker, Lamberts, & van Rossum, 2009; van Rossum, et al., 2003), although the mechanisms are unknown. When the expanded haplotype 2 (Cao-Lei, et al., 2011) is considered, around half of the carriers should also carry the minor alleles of the functional rs3806855 and rs3806854 in promoter 1B, and rs10482614 in promoter 1H. *In vitro* all three minor alleles reduced promoter 1H and 1B activity between 50 and 80% (Cao-Lei, et al., 2011). Methylation of the

entire 1B or 1H promoter had a similar effect, reducing promoter activity by up-to 90%. Logically, carrying haplotype 2 or having high promoter 1H methylation would have similar consequences including lower GR levels, increased the cardiovascular stress reactivity and activity. Although only methylation level was associated with differential cardiovascular responses to SeCPT, whereas *Bcl*I / haplotype 2 influenced heart rate independent of experimental group, there will be overlap in the mechanisms underlying their actions. Nevertheless, SNPs in a high LD with those investigated in this study might be regulators of methylation and physiological traits, especially since genetic variation that leads to methylation and expression variation at the same locus is not a rare phenomenon (Wagner et al., 2014). We hypothesise that the decreased promoter methylation observed in haplotype 2 carriers represents a counterbalance to the potential deleterious effects of the *Bcl*I genotype.

There is a well-established genetic component to variability in DNA methylation. Methylation Quantitative Trait Loci (mQTL) are single genetic variants, often SNPs that correlate, or are associated with, DNA methylation levels. mQTLs have operate over distances as large as 5 kb, occurring for approximately 2% of the measured CpGs, and 9.5% of the expressed regions (Wagner, et al., 2014). In contrast to Bromer *et al.* (Bromer, et al., 2013), we observed the *Bcl*I minor allele to correlate with high sum promoter 1F methylation levels. In our linear mixed effect model of the stress response there was a significant interaction between methylation, genotype, and cardiovascular activity. Sum methylation levels for promoter 1H and 1F+1H were higher in women than men, and methylation levels were not normally distributed in either sex. Sex-specific DNA methylation profiles were not unexpected as genome-wide levels are known to be higher in males (Fuke et al., 2004; Shimabukuro et al., 2007). However, locus specific increases are not limited to males, but have also been reported for women (Eckhardt et al., 2006; El-Maarri et al., 2007; Sandovici et al., 2005; Sarter et al., 2005). Similarly, increased age has been linked to both a reduction in global methylation levels, and dramatic genome-wide redistributions of 5-mC (Liu et al., 2011). However, given the narrow age distribution of our participants, this was not observed.

Although only the methylation level was associated with differential cardiovascular responses to SeCPT, whereas *Bcl*I influenced heart rate independent of the experimental group, there might be a functional overlap between the two. Haplotype 2 and increased 1H methylation would both be expected to decrease promoter activity, not only representing a specific *GR* mQTL, but also an expression methylation quantitative trait locus (emQTL) and even further a physiological expression methylation trait locus integrating the cardiovascular and stress responses with both genetic variants and methylation levels. Previous emQTL reports have all covered single CpG dinucleotides. There is currently contradicting data on the functional relevance of such limited methylation changes (Vinkers et al., 2015). For the *GR*, we have previously shown that complete methylation throughout each proximal *GR* promoter efficiently inactivates them (Cao-Lei, et al., 2011). Similarly, methylation of a smaller (around 125 bp) fragment containing multiple CpGs also has functional effects, reducing promoter activity to ~25% of the control, unmethylated, sequence (McGowan, et al., 2009). However, there is currently no evidence that methylation of a single CpG has functional consequences on *GR* expression. The importance of promoter wide changes in DNA methylation is supported by recent clinical data from subjects suffering from posttraumatic stress disorder (PTSD). Whilst Lebonté *et al*. identified two CpGs in *GR* promoter 1F that associated with PTSD, Yehuda *et al*. nicely demonstrated that changes occurred promoter-wide (Labonte, Azoulay, Yerko, Turecki, & Brunet, 2014; Yehuda, et al., 2015). This is mirrored in both the rodent maternal care paradigm and the healthy human brain. Screening chromosome 18 that contains the rat GR, differential DNA methylation was observed in clusters across broad genomic regions (Suderman et al., 2012). At the individual CpG dinucleotide level strong distance-dependent correlations were found (Cao-Lei, et al., 2013), further supporting our interpretation that DNA methylation changes occur in clusters, and levels at individual CpGs are inter-dependent. These data lead us to suggest that our emQTL, unlike previous reports, is between haplotype 2 and a functionally relevant cluster of methylated CpGs in promoter 1H some 3 kbp upstream of the investigated region.

The generalizability and relevance of DNA methylation in peripheral blood samples to other tissues may appear questionable, as patterns are both locus and tissue specific. However, depending on the origin of the methylation patterns, it is probable that peripheral blood methylation levels are epigenetic proxies that mirror patterns in individual tissues of the cardiovascular system or the HPA axis. There are two plausible, non-exclusive, mechanisms for this. Firstly, peripheral epigenetic variations may be the results of systemically acting circulating epigenetic modifiers such as cortisol (Guintivano & Kaminsky, 2014). Secondly, they may originate from a commonly programmed developmental precursor tissue. DNA methylation is established *de novo* during embryogenesis, when it is particularly susceptible to environmental influences. Epigenetic changes across primary germ layers occurring in this period will result in levels common to several differentiated tissues (Petronis, 2006). We have observed a strong correlation in methylation levels between ectoderm-derived tissues such as the anterior pituitary and the adrenal gland (Witzmann, et al., 2012), as well as throughout the different neural tube derived tissues throughout the human brain (Cao-Lei, et al., 2013) supporting the latter hypothesis. The corollary to this is that peripheral methylation levels may also be proxies for the functional difference in GC sensitivity in other tissues from the same developmental origins.

The observation that haplotypes 2 and 3 have specific and different cardiovascular effects suggests that they act through different pathways. This concords with prior evidence that the renal pressure-natriuresis system and acute sympathetic activation mechanisms influence baseline cardiovascular traits and cardiovascular reactivity respectively (Busjahn, Faulhaber, Viken, Rose, & Luft, 1996). However, the role of GC and the GR in these mechanisms is unclear. In GC induced hypertension, pharmacological stimulation and receptor blocking data exclude direct GC/GR interactions (Hattori et al., 2013; Whitworth, Mangos, & Kelly, 2000), suggesting indirect mechanisms such as oxidative stress or nitric oxide deficiency (Ong, Zhang, & Whitworth, 2008; Plomin et al., 2013). Nevertheless *in vitro* GC have significant effects on the NO system, including reducing endothelial and inducible NOS levels, reducing L-arginine and co-factor availability as well as inhibition of transmembrane L-arginine

transport (Radomski, Palmer, & Moncada, 1990; Simmons et al., 1996). Our data confirm this link between GC/GR and the cardiovascular response, albeit potentially via an indirect mechanism. There was a very clear link between SBP, to a lesser extent DBP, and methylation of promoter 1B and 1H. This was confirmed by the observation that *GR* haplotype 3 carriers had lower blood pressure after SeCPT and a higher heart rate decrease. This implies that carriers of *GR* haplotype 3 may be protected against hypertension to some extent, even if there does not appear to be direct GC/GR involvement. Inversely, *GR* haplotype 2 had a significantly higher baseline heart rate. Indeed, the constituent *Bcl*I has been linked to hypertension (J. U. Weaver, Hitman, & Kopelman, 1992). Cortisol secretion (AUCg) was increased uniquely in carriers of the *Bcl*I containing haplotype 2. Our observations on the functional effects of haplotype 2 and 3 can be generalised from our highly homogenous population to other populations, as these haplotypes have identical functional effects irrespective of ethnicity (Melcescu et al., 2012).

A weakness of our study is the limited number of SNPs that were investigated; however, the principal haplotypes previously established in the literature were readily identified. Similarly, the relatively small number of donors was counterbalanced by the high heterogeneity of the young, infrequent-smoking, undergraduate student population, reducing confounding socioeconomic factors.

3.6 Conclusions

This is one of the first studies linking epigenetic modifications of the GR promoter, receptor genotype and physiological measures of the stress response. In the baseline period prior to the water task, there were clear genetic and epigenetic effects on blood pressure, particularly the *Bcl*I containing haplotype 2 and promoter 1F and 1H methylation. This was independent of the experimental group. The water task induced a strong cardiovascular and HPA axis response in the SeCPT group and both systems were affected principally by the functional genetic variants. Methylation predicted lower SBP and DBP evolution over time in response to the water task. The return to baseline was predominantly influenced by the genomic sequence. The *Bcl*I polymorphism was associated with promoter 1H methylation levels.

Promoter 1F methylation levels did not associate with any of the observed genetic variants, and as such are potentially influenced by the environment. Overall, we have shown that the induction and resolution of the stress response is controlled by an exquisite mix of genetic and epigenetic factors.

SM-Table 3.1 Commonly reported haplotypes

Haplotype	**Genotype Groups**	Previously Reported Haplotype Name / Number							
		a	b	c	d	e	f	g	h
Haplotype 1	Most Common Haplotype (MCH)	WT	1	1	1	1	1	1	1
Haplotype 2	*Bcl*I alone	*Bcl*I	4	4	2	1	4-1	2	2
Haplotype 3	*TthIII*I + NR3C1-I + *9beta*			5		3	5	4	3
Haplotype 4	*Bcl*I + *ThIII*I + 1H	*ThIII*I + *Bcl*I				2	4-2	3	4
Haplotype 5	*ThIII*I + NR3C1-I + 9 beta + E22/23K	*ThIII*I + 9 beta + E22/23K	2	2	4	4	2	5	6
Haplotype 6	*ThIII*I alone	*ThIII*I							

[a]:Van Rossum, E.F.C., et al., Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms. Clinical Endocrinology, 2004. 61(5): p. 573-581.

[b]: Kumsta, R., et al., Sex specific associations between common glucocorticoid receptor gene variants and hypothalamic-pituitary-adrenal axis responses to psychosocial stress. Biological Psychiatry, 2007. 62(8): p. 863-869.

[c]: Kumsta, R., et al., Characterization of a glucocorticoid receptor gene (GR, NR3C1) promoter polymorphism reveals functionality and extends a haplotype with putative clinical relevance. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2009. 150B(4): p. 476-482.

[d]: Otte, C., et al., Glucocorticoid receptor gene and depression in patients with coronary heart disease: The Heart and Soul Study—2009 Curt Richter Award Winner. Psychoneuroendocrinology, 2009. 34(10): p. 1574-1581.

[e]: Dekker, M.J.H.J., et al., Effect of glucocorticoid receptor gene polymorphisms in Guillain-Barré syndrome. Journal of the Peripheral Nervous System, 2009. 14(2): p. 75-83.

[f]: Cao-Lei, L., et al., Transcriptional control of the human glucocorticoid receptor: identification and analysis of alternative promoter regions. Human Genetics, 2011. 129(5): p. 533-543.

[g]: Spijker, A.T., et al., Glucocorticoid and mineralocorticoid receptor polymorphisms and clinical characteristics in bipolar disorder patients. Psychoneuroendocrinology, 2011. 36(10): p. 1460-1469.

[h]: Hardeveld, F., et al., Glucocorticoid and mineralocorticoid receptor polymorphisms and recurrence of major depressive disorder. Psychoneuroendocrinology, 2015. 55: p. 154-163

SM-Table 3.2: *GR* SNPs PCR primers and their reaction condition.

SNPs name	Sequence[a]	Tm[b] (ºC)	Mg^{2+} (mM)	Primers (µM)
*Tth*III*I*	Fwd: 5'gtatttgttgggtgcctgct-3' Rev: 5'actccagtgtgccagaaagg-3'	57.7	2.5	0.3
NR3C1-I	Fwd: 5'-aactcggtggccctcttaac-3' Rev: 5'-aacctgttggtgacgcttg-3'	56.2	1.5	0.4
1H	Fwd: 5'-gccagaggtaagaagcgaggcggga-3' Rev: 5'-gccggggcctccccggagcc-3'	70	0.7	0.1
ER22	Fwd: 5'-cagccgtgattgaaaagagg-3' Rev: 5'-gccttttggaaaatcaacca-3'	56.3	2.0	0.3
EK23	Fwd: 5'-cagccgtgattgaaaagagg-3' Rev: 5'-gccttttggaaaatcaacca-3'	56.3	2.0	0.3
*Bcl*I	Fwd: 5'-ttgctaaagcaatgcagtgaa-3' Rev: 5'-tcaaacgaaagctgaaaattga-3'	57.7	2.5	0.4
9beta	Fwd: 5'tgactcctgtttaaaaataaaagttg-3' Rev: 5'-cagattggacaatcggaactg-3'	57.7	2.5	0.4

[a]Fwd, forward or sense primer; Rev, reverse or antisense primer. Primer with restriction enzyme recognition sites (underlined) used for amplification of the promoter fragments.

[b]Tm, annealing temperature in PCR.

SM_Table 3.3: Association of *GR* gene SNPs and Haplotypes with the response of blood pressure, heart rate and cortisol to the SeCPT.

Genotypes and Haplotypes	***TthIII*I**		**NR3C1-1**		**1H**		**E22E**		**R23K**		***BclI* (CC vs GG)**		**9beta**		**Haplotype1_CTGGGCA**		**Haplotype2_CTGGGGA**		**Haplotype3_TCGGGCG**		**Haplotype4_TTAGGGA**		**Haplotype5_TCGAACG**		**Haplotype6_TTGGGGA**	
	maineffect[a]	interaction[b]	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction	maineffect	interaction
Blood pressure																										
SBP_basline												0,096						0,048							0,083	
SBP_recovery												0,075						0,067		0,019						
SBP_peak																										
SBP_ increase																						0,075				
SBP_ decrease																										
Heart rate																										
bpm_basline											0,048						0,022									
bpm_recovery											0,061						0,027									
bpm_peak																	0,023									
bpm_ increase												0,029														
bpm_ decrease																			0,016							
Cortisol response																										
AUCG																	0,053	0.034								
AUCI																										

[a] genotypes or haplotypes as maineffect factor

[b] For blood pressure and heart rate, the interaction is between genotypes or haplotypes and SeCPT group. For cortisol response, the interaction is between genotypes or haplotypes, gender and SeCPT group.

P values are: unfilled squares p>0.1; hatched squares 0.1>p>0.05; filled squares p<0.05**.**

3.7 Acknowledgements

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Chapter 4

Promoter haplotypes of the corticotropin-releasing hormone encoding gene modulate the physiological stress response *in vitro* and *in vivo*

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4.1 Abstract

The corticotropin-releasing hormone (CRH) is a neuropeptide mediating stress responses. CRH exerts effects via the hypothalamic-pituitary-adrenal axis as well as immediate effects on the sympathetic-adrenal-medullary system. Genetic variants of the *CRH* promoter were previously found to be associated with altered *CRH* promoter activity and physiological reactions.

Functional characterization of three *CRH* promoter haplotypes have been performed *in vitro* using a reporter gene assay under different stimulation conditions. Furthermore, 232 healthy subjects were genotyped and the influence of *CRH* haplotypes on basal parameters such as post-awakening cortisol and blood pressure as well as on stress reactivity measured after socially evaluated cold pressor test (SeCPT) was investigated.

In vitro, CRH haplotype 2 showed the highest promoter activity under baseline conditions and after forskolin stimulation compared to other haplotypes. Forskolin treatment resulted in a two-fold increase of haplotype 2 promoter activity compared to the baseline condition. Cell line-dependent promoter activation was found after hydrocortisone treatment. *In vivo*, *CRH* haplotype 2 carriers showed significantly higher baseline blood pressure ($p = 0.002$) and blood pressure after SeCPT ($p < 0.001$), but did not differ in cortisol levels.

This study provides converging evidence for the importance of *CRH* promoter variants on physiological stress response parameters.

4.2 Introduction

The physiological stress system includes the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary system (SAM) (Eysenck, 2004). Parameters representing these systems such as cortisol, blood pressure or heart rate define the physiological body reaction in response to a stressor. The corticotropin-releasing hormone (CRH), a 41-amino acid peptide, regulates both systems at different anatomical levels. As an immediate response to a stressor, CRH acts on the cardiovascular system as a non-hypophyseotropic hormone through nerve impulses (Kovács, 2013; Kronenberg et al., 2007). In addition, CRH can also cope with stressors as a hypophyseotropic hormone through the initiation of the endocrine cascade of the HPA axis (Kalogeras et al., 1996; Kovács, 2013; Kronenberg et al., 2007). Regulation and function of hypophyseotropic/non-hypophyseotropic CRH might depend on involved CRH receptors (CRHR1/2), CRH binding protein (CRHBP) or steroid receptor coactivators (SRC-1a/e) (Kovács, 2013; Kronenberg et al., 2007). Further, susceptibility to situational and individual influences (Kudielka et al., 2009), and the contribution of genetic variances needs consideration.

The human *CRH* gene contains two exons encoding the CRH pre-prohormone (Arbiser et al., 1988). Two single nucleotide polymorphisms (SNPs), rs3176921 (T/C) and rs5030875 (T/G), located in the 5'-regulatory region of the *CRH* locus, have been described, assigned to three haplotypes A1B1 (haplotype 1), A2B2 (haplotype 2) and A2B1 (haplotype 3) (Baerwald et al., 1999; Baerwald et al., 1996) and used in a few number of association studies (Baerwald et al., 1999; Baerwald et al., 1996; Wagner et al., 2006). The minor G allele of rs5030875 was associated with higher stress-induced HPA axis function in patients with rheumatoid arthritis (Malysheva et al., 2011) but not with cortisol secretion or sensitivity in healthy men (Rosmond et al., 2001). The minor C allele of rs3176921 was associated with higher verbal intelligence and cognition values in healthy adults (Strohner, 2011), but not investigated with respect to stress. So far, only one study analyzed SNPs of *CRH* with respect to blood pressure, heart rate or cortisol response following stress. The rare allele of rs5030875 showed a significant interaction

with a functional SNP (*ThtIII*I) of the glucocorticoid receptor gene (*GR, NR3C1*), but no effects on diurnal saliva cortisol variation or dexamethasone suppression (Rosmond et al., 2001). Haplotypes of *CRH* have not yet been investigated in a cohort tested in a standardized laboratory stress test.

The aim of the present study was to characterize *CRH* promoter haplotypes using a luciferase reporter gene assay *in vitro.* Following previous findings on *CRH* promoter functionality (Rosmond et al., 2001; Wagner et al., 2006) forskolin and hydrocortisone were considered as stimuli for better comparison between *in vitro* and *in vivo* markers of stress reactivity. For this purpose, an association study with *CRH* haplotypes, post-awakening cortisol, and parameters of a laboratory-induced stress response in 232 healthy subjects (cf. (Li-Tempel et al., 2016a; Li-Tempel et al., 2016b)) was performed. We assumed that *CRH* haplotypes modulate promoter activity and *in vivo* stress responses in a corresponding manner. Haplotypes resulting in a strong promoter activation are hypothesized to show association with physiological parameters and/or stress reactivity.

4.3 Material and Methods

Functional characterization of *CRH* promoter SNPs *in vitro*

Construction of h*CRH* reporter plasmids

To analyze the potentially altered promoter activity of the human *CRH* gene, 3651 bp corresponding to the human *CRH* promoter region were amplified with PCR using genomic DNA as template and sub-cloned in a sense orientation (antisense was used as a negative control) upstream of *luc*+ into the luciferase reporter vector pGL3-basic (Promega Corporation, Mannheim, Germany) between *Xma*I and *Eco53*kI (NEB, R0180 and R0116, Frankfurt a. M., Germany). The 3651 bp DNA fragments were confirmed by sequencing and contain a set of four polymorphisms of which two were examined more closely. The SNP rs3176921 (T/C) at position -684 bp co-segregate with the SNPs rs5030877 and rs5030876 resulting in two alleles (A1/A2) and was selected for further investigations. The biallelic polymorphism rs5030875 (T/G) at position -3371 bp correspond to alleles B1/B2. All positions refer to the transcription start site of the *CRH* gene. Finally, three different haplotypes could be assigned, haplotype 1 (T-T), haplotype 2 (G-C) and haplotype 3 (T-C) (Figure 4.1). Haplotype 1 correspond to haplotype A1B1, haplotype 2 to A2B2 and haplotype 3 to A2B1 in the study of Wagner and colleagues (2006). Detailed information on plasmid construction is available in SM-Table 4.1 and SM-Figure 4.1.

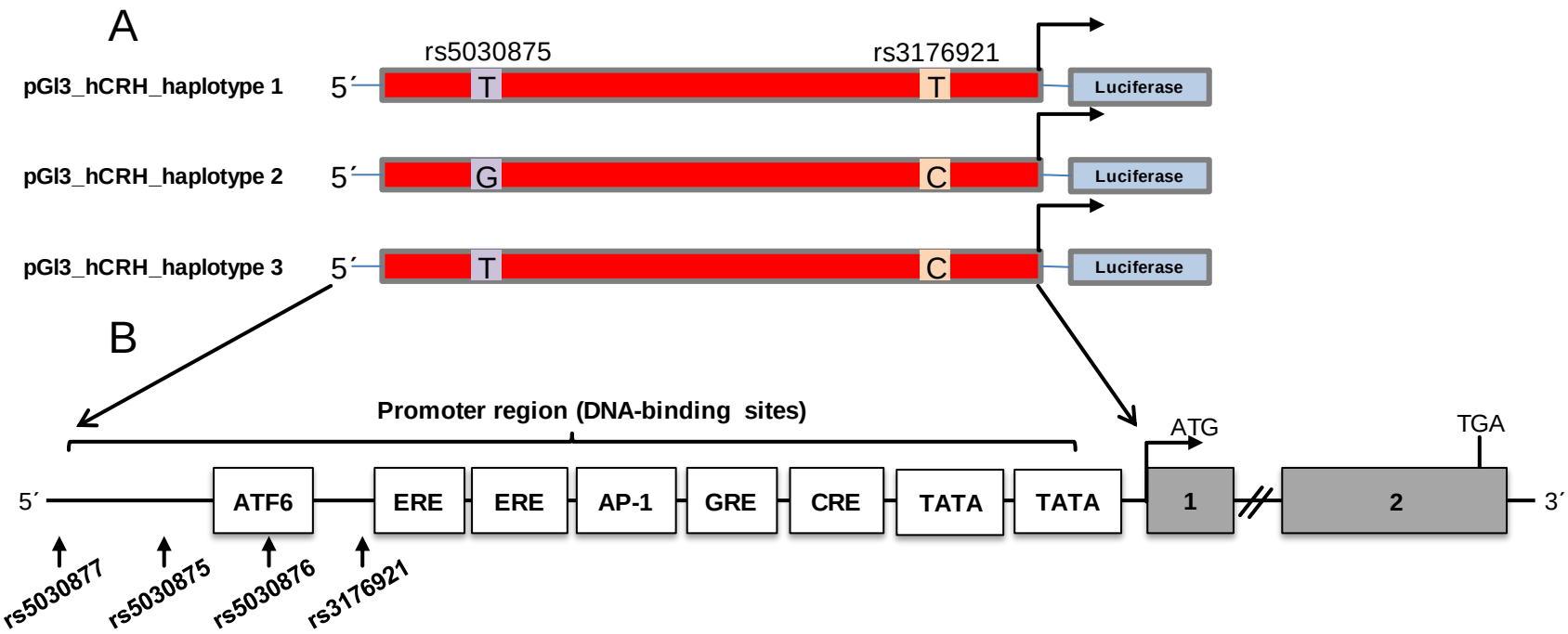


Figure 4.1. A: Schematic representation of pGl3 constructs containing 3651 bp of the human *CRH* promoter sequence. The single nucleotide polymorphisms (SNPs) rs503875 (T/G) and rs3176921 (T/C) in the *CRH* promoter are highlighted in boxes. Combination of both SNPs results in three haplotypes. The arrow represents the transcription start site. B: Location of studied *CRH* SNPs in the promoter region and diagrammatic representation of the *CRH* promoter showing the sequential occurrence of response elements that are thought to have functional role regulating gene expression. (The location in the figure is not in relative correspondence to the actual sequence size).

Cell culture, transfection, stimulation and luciferase assay

The mouse pituitary corticotroph tumor-derived cell line AtT−20/D16v−F2 (AtT−20) was kindly donated by Christoph Baerwald (Department of Rheumatology, University Hospital, Leipzig, Germany). The AtT-20 cells were grown under standard culture conditions in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g D-glucose/ml without L-glutamine phenol red, Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (FBS, Lonza), 2 mM L−glutamine and 0.1 mg/ml penicillin/streptomycin (Pen/Strep, Sigma, Steinheim, Germany). The human glioblastoma–astrocytoma U373MG cells (ECACC 89081403) were grown in Eagle's Minimal Essential Medium (EMEM, Lonza), supplemented with 10% FBS (Lonza), 2 mM L-glutamine (Lonza), and 1% NEAA (Lonza). Both cells were maintained in the in 37 °C incubator containing 5% CO_2.

AtT-20 Cells and U373MG were transfected using Lipofectamin 3000 transfection reagent (InvitrogenTM, life technologies, California, United States) following the manufactures instruction. Briefly, cells were passaged at 80 % confluency and seeded in a density of $3x10^5$cells/ml in 6-well plates. After 24 h of incubation, 3 µg DNA (2.98 µg Firefly luciferase constructs and 0.02 µg pGL4.74 ([HRLUC/TK], Renilla Luciferase; Promega) were added to the cell culture. Since the regulation of *CRH* gene expression might be affected by the transcription factors (TFs), cAMP response element-binding protein (CREB) and GR, we determined the promoter activity in response to forskolin and hydrocortisone. Both stimulants (in contrast to 8-Br-cAMP and dexamethasone) were selected due to their better physiological compatibility to *in vivo* studies. Time course and doses below were chosen after reviewing previous studies (Guardiola-Diaz et al., 1996; Reisine et al., 1986). The ratio of the dosage conversion of dexamethasone to hydrocortisone was 25 and calculated using an online tool (http://clincalc.com/Corticosteroids/). 24 h after transfection, 50 µM forskolin (Sigma) or 2.7 µM hydrocortisone (Sigma) was added and cells were incubated for another 24 h. Cells were collected and subjected to passive lysis. Luciferase activity was detected by spectrometry (Berthold, Bad Wildbad, Germany) using the Dual-Luciferase Reporter Assay (DRL) system (Promega), in which Renilla luciferase activity was used to normalize the

transfection efficiency. The intensity of the measurement varied between 500 to 5000 light units and the background signal of 6 to 8 light units was subtracted, respectively. Promoter activities are presented as relative light units (RLUs), calculated as the quotient of the individual Firefly luciferase driven value (promoter activity) divided by the corresponding Renilla luciferase driven value (transfection efficiency control). As controls, pGL3-control, empty pGL3-basic vectors and antisense direction constructs were used.

Genetic association study *in vivo*

Participants

232 participants were recruited from the University of Trier as previously reported (Larra et al., 2014; Li-Tempel et al., 2016a; Li-Tempel et al., 2016b). Briefly, 218 healthy men and women (115 females, mean age years = 23 ± 2.8; BMI 22.4 ± 0.25) completed the experimental protocol. Participants with an increased objective or subjective sensitivity to cold, any indication of circulatory disturbances, cardiovascular problems, smoking more than five cigarettes per day or alcohol abuse were excluded. Participants had to be medication free, contraceptives (except ethinylestradiol and drospirenone containing ones such as Yasmin (Bayer-Schering Pharma, Leverkusen, Germany) were allowed at the time of testing as studies showed no influence of contraceptives on the cortisol awakening response (Wüst et al., 2000). As previously reported, caffeinated and alcoholic drinks, physical exercise, and meals were not permitted in the 3 hours immediately preceding the experimental visit. The protocol was approved by the ethics committee of the state's medical association (Landesärztekammer Rheinland-Pfalz) and in accordance with the declaration of Helsinki. All participants gave written informed consent.

DNA extraction, genotyping and haplotype construction

DNA was extracted from 10 ml EDTA blood following a standard NaCl salting out method according to the protocol of Miller (Miller et al., 1988). The *CRH* SNPs rs3176921 and rs5030875 were amplified using standard PCR method. For allelic discrimination, the amplified PCR products of rs3176921 and rs5030875 were subsequently digested with the

restriction enzyme *Bsm*AI or *Xmn*I, respectively. Detailed information about primer sequences and PCR conditions are available in supplementary information (SM-Table 4.2).

Assessment of post-awakening cortisol

Participants were asked to collect at five times saliva samples using Salivettes (Sarstedt, Nümbrecht, Germany) at home; immediately after awakening and 15, 30, 45 and 60 min thereafter on two consecutive weekdays for the assessment of post-awakening cortisol. They were instructed to abstain from food, drinks other than water and brushing their teeth before completion of saliva sampling. Furthermore and for reasons of compliance, they were told to pay special attention to keep the timing right to ensure the reliability of their data. Salivary cortisol was analyzed as previously reported (Li-Tempel et al., 2016b), in correspondence with consensus guidelines for the assessment of post-awakening cortisol (Stalder et al., 2016).

Socially evaluated cold pressor test (SeCPT)

The SeCPT was performed as previously reported (Lass-Hennemann et al., 2011; Schwabe et al., 2008). Briefly, participants assigned to the SeCPT group were asked to completely immerse their hand in ice-cold (2-3 °C) water. Participants assigned to the control group were asked to completely immerse their hand in isothermic (35-37 °C) water. Participants in the SeCPT group were under the social surveillance of an experimenter; their perceptions of social evaluation, uncertainty and lack of control were enhanced by warning them that the procedure may be painful, not communicating the duration of immersion during the test, and informing them that their performance would be recorded for subsequent facial expression analysis. Participants assigned to the control group were not under social surveillance and no video camera was present. All participants were asked to remove their hand from the water after 3 minutes. Heart rate (HR) and systolic, diastolic blood pressure (SBP, DBP) were measured throughout the experiment using the Dinamap System (Critikon; Tampa, Florida, USA) with the cuff placed on the right upper arm. Assessments at three times were used to calculate the recovery period: 10 mins, 25 mins, and 55 mins after SeCPT finished.

Cortisol, measured in salivary samples collected at five time points (one time pre SeCPT and four times post SeCPT), served as an indicator for HPA axis reactivity.

Statistical analysis

Group comparisons in the *in vitro* studies were performed using an analysis of variance (ANOVA). Both SNPs were tested for Hardy-Weinberg equilibrium (HWE). Haploview 4.2 (Barrett et al., 2005) was used to assess inter-marker linkage disequilibrium scores (LD scores), expressed as D' and r^2. Individual haplotypes were reconstructed using the program PHASE, version 2.1 [(Stephens et al., 2001) http://stephenslab.uchicago.edu/software.html#phase], which uses an algorithm based on coalescence-based Bayesian haplotype inference for predicting haplotypes from genotype data, combining modeling strategy with computational strategies. Post-awakening cortisol levels assessed on two consecutive days were averaged in order to enhance the reliability of the measure (Hellhammer et al., 2007). The area under the curve with respect to ground (AUCg) and increase (AUCi) was calculated as previously reported (Pruessner et al., 2003). Briefly, the formula for five times measures for AUCg = $\sum_{i=1}^{n-1} \frac{(m_{(i+1)}+m_i)}{2}$ and AUCi = $\left(\sum_{i=1}^{n-1} \frac{m_{(i+1)}+m_i}{2}\right) - (n-1)m_i$ ($m_i$denoting the individual measurement, and n the total number of measurments). AUCg and AUCi served as dependent variables in the subsequent analyses. Cardiovascular data (HR, SBP, DBP) were reduced by extracting the mean increase of dependent variables from baseline to the peak after the water task and mean decrease from the peak to the recovery period. These are referred to as e.g. "SBP peak", "SBP increase" and "SBP decrease". Baseline was considered as an average value of three measurements before water task. Recovery period was calculated using the three measurements after the water task. Results were considered statistical significant with a p-value < 0.05. The threshold of significance was set at $p < 0.05$, *, ** and *** represent $p < 0.05$, $p< 0.01$ and $p< 0.001$, respectively. Statistical analyses were performed using SPSS 20.0. Analyzed data are indicated as mean ± SEM.

4.4 Results

Functional characterization of *CRH* haplotypes *in vitro*

Baseline condition

The luciferase activity for three *CRH* promoter constructs under baseline and stimulated conditions in two cell lines (AtT-20 and U373MG) is shown in Figure 4.2 A, E. In both cell lines, the promoter activity was highest for haplotype 2. In detail, comparison of transcriptional activity between the haplotypes measured in the AtT-20 cell line revealed significantly higher transcriptional activity of haplotype 2 compared to haplotype 1 [t (16) = 8.85, p< 0.001)], and haplotype 1 showed significantly higher transcriptional activity than haplotype 3 under unstimulated conditions (basal) [t (16) = 5.07, p< 0.001]. In the U373MG cell line, haplotype 2 showed significantly higher transcriptional activity compared to haplotype 1 [t (16) = 3.867, p = 0.001], as well as compared to haplotype 3 [t (16) = 2.572, p = 0.020], whereas haplotype 1 and haplotype 3 did not differ significantly [t (16) = 0.785, p = 0.444].

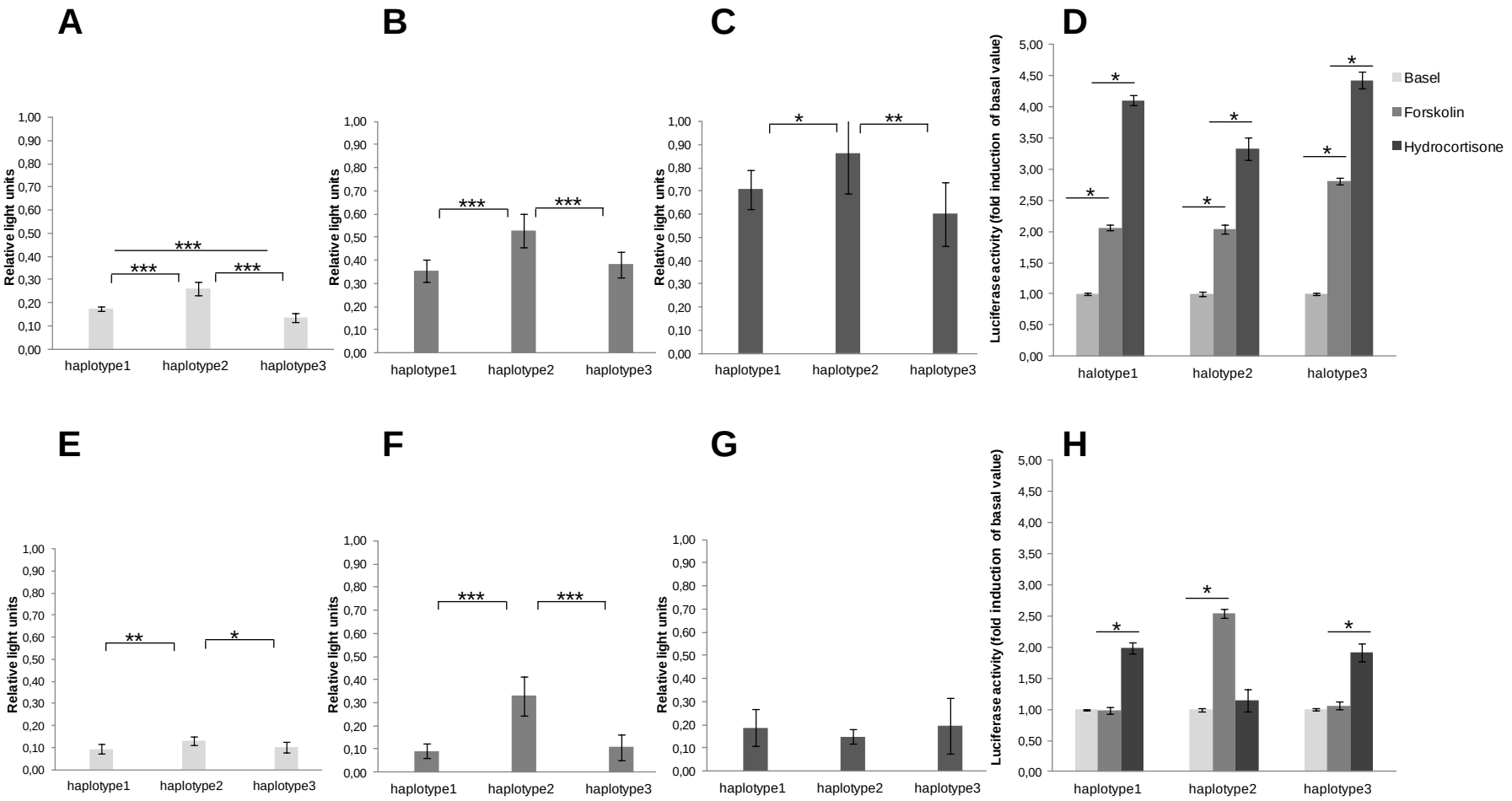


Figure 4.2. Activity of different *CRH* promoter alleles (haplotypes 1, 2, and 3) measured by luciferase activity in the AtT-20 cell line (A–D) and the U373MG cell line (E-H). Cells were treated with forskolin (50uM) and hydrocortisone (2.7uM) for 24 h before subjected to dual luciferase reporter assay. A) AtT-20 cells in control condition. B) AtT-20 cells under forskolin stimulation. C) AtT-20 cells under hydrocortisone stimulation. E) U373MG cells in the control condition. F) U373MG cells under forskolin stimulation. G) U373MG cells under hydrocortisone stimulation. Fold induction of luciferase activity of three haplotypes with respect to the control condition in AtT-20 (D) and U373MG cells (H). The results are represented as the mean ± SEM of three independent experiments performed in triplicate (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Stimulation condition

We used a three (haplotype: 1, 2, 3) x three (condition: basal, forskolin, hydrocortisone) ANOVA to examine the promoter activity in AtT-20 cells. In addition to significant main effects of haplotype, F (2, 16) = 309.88, p< 0.001, and of condition, F (2, 16) = 92.48, p< 0.001, the interaction was significant, F (4, 32) = 4.01, p = 0.010. Transcriptional activity increased for all three haplotypes constructs after stimulation with forskolin or hydrocortisone (Figure 4.2 B,C). A detailed overview of pairwise comparisons of all significant results is given in Table 1. Comparison of transcriptional activity between the haplotypes in the AtT-20 cell line revealed a significantly higher transcriptional activity of haplotype 2 compared to haplotype 1 as well as compared to haplotype 3 if stimulated with forskolin or hydrocortisone whereas haplotype 1 and haplotype 3 did not differ significantly (see Table 4.1).

Table 4.1: Pairwise comparison of promoter activity between three *CRH* promoter haplotypes in two cell lines under basal and stimulation condition.

	AtT-20		**U373MG**	
	t	*p*	t	*p*
Haplotype 1				
Basal vs. For	11.382	< 0.001	0.135	0.894
Basal vs. HC	18.802	< 0.001	3.461	0.003
For vs. HC	10.886	< 0.001	3.392	0.004
Haplotype 2				
Basal vs. For	10.459	< 0.001	6.963	< 0.001
Basal vs. HC	10.324	< 0.001	1.438	0.174
For vs. HC	5.354	< 0.001	5.001	< 0.001
Haplotype 3				
Basal vs. For	12.573	< 0.001	0.320	0.753
Basal vs. HC	10.053	< 0.001	2.283	0.036
For vs. HC	4.441	< 0.001	1.971	0.066
Basal				
haplotype 1 vs. haplotype 2	8.847	< 0.001	3.867	0.001
haplotype 1 vs. haplotype 3	5.073	< 0.001	0.785	0.444
haplotype 2 vs. haplotype 3	11.036	< 0.001	2.572	0.020
Forsokolin				
haplotype 1 vs. haplotype 2	6.072	< 0.001	7.979	< 0.001
haplotype 1 vs. haplotype 3	1.097	0.289	0.787	0.446
haplotype 2 vs. haplotype 3	4.868	< 0.001	6.576	< 0.001
Hydrocortisone				
haplotype 1 vs. haplotype 2	2.449	0.026	1.141	0.274
haplotype 1 vs. haplotype 3	1.957	0.068	0.182	0.858
haplotype 2 vs. haplotype 3	3.564	0.003	0.935	0.367

Abbreviations: For: Forskolin; HC: Hydrocortisone

Another three (haplotype) x three (condition) ANOVA examined promoter activity in the U373MG cell line. In addition to significant main effects of haplotype, F (2, 10) = 19.13, p < 0.001, and of condition, F (2, 10) = 20.72, p< 0.001, the interaction was significant, F (4, 20) = 8.35, p = 0.010. Forskolin increased transcriptional activity for haplotype 2 but not haplotypes 1 and 3 (Figure 4.2 F). In contrast, hydrocortisone increased the promoter activity for haplotypes 1 and 3 but not haplotype 2 (Figure 4.2 G). Comparison of transcriptional activity between the haplotypes revealed that under stimulation with forskolin, haplotype 2 showed significantly higher transcriptional activity compared to haplotype 1 as well as compared to haplotype 3 whereas haplotype 1 and haplotype 3 did not differ significantly. Under hydrocortisone stimulation, there were no significant differences between haplotypes. All pairwise comparisons can be found in Table 4.1.

Comparison of forskolin and hydrocortisone stimulation relative to baseline is depicted in Figure 4.2 D, H. In AtT-20 cells, treatment with hydrocortisone resulted in a higher increase in luciferase activity compared to forskolin. This effect was found for all three *CRH* haplotypes (Figure 4.2D). However, the fold increase to baseline varied with 4.424 ± 0.138 times (p< 0.001) in haplotype 3, 4.107 ± 0.848 times (p< 0.001) in haplotype 2, and 3.326 ± 0.174 times (p< 0.001) in haplotype 1. In contrast, this pattern did not occur in U373MG cells (Figure 4.2H).

Genetic association study *in vivo*

Sample characteristics

A relatively homogenous cohort of 232 undergraduate students with minimal lifestyle differences, determined from exclusion and inclusion criteria, were recruited, and data from 218 (103 males and 115 females) were analyzed (SM-Table 4.3).

Two *CRH* promoter polymorphisms, rs5030875 and rs3176921, were analyzed and an overview of the genetic data is given in the supplements (SM-Table 4.4). In line with the literature, both *CRH* SNPs were in high linkage disequilibrium (D' = 1, r^2= 0.48). The

haplotype structure was successfully created with PHASE. *CRH* haplotype 1 (T-T) had a frequency of 89.7% and consisted of the major alleles of each SNP. Haplotype 2 (G-C) with a frequency of 5.3% contained the minor alleles of each SNP. Haplotype 3 (T-C) showed a frequency of 5.0%. The haplotype frequencies were similar to those previously reported (Wagner et al., 2006). For the *CRH* genotypes and haplotypes, there were no differences in the distributions with respect to age and gender.

Basal condition

Post-awakening cortisol is used as an indicator for the basal state of the HPA axis. The average value of three repeated measures of blood pressure and heart rate before the SeCPT experiment served as cardiovascular parameters for the basal sympathetic-adrenal-medullary system (SAM) state. To evaluate the influences of CRH haplotypes on post-awakening cortisol expressed as AUCi and AUCg as well as baseline blood pressure and heart rate, a series of one-way ANOVAs were performed (Table 4.2). Haplotype 2 had a significant effect on baseline SBP [$F(1, 216) = 9.696$, $p = 0.002$]. Carriers of haplotype 2 had higher SBP compare to non-carriers (mean 121.1 ± 2.56; mean 112.732 ± 0.818). In addition, an association of haplotype 3 and AUCg approached significance [$F(1, 215) = 3.883$, $p = 0.05$] with higher AUC_g in carriers of haplotype 3 compared to non-carriers (mean 54.715 ± 4.253; mean 45.92 ± 1.355). Statistically significant results are depicted in Figure 4.3.

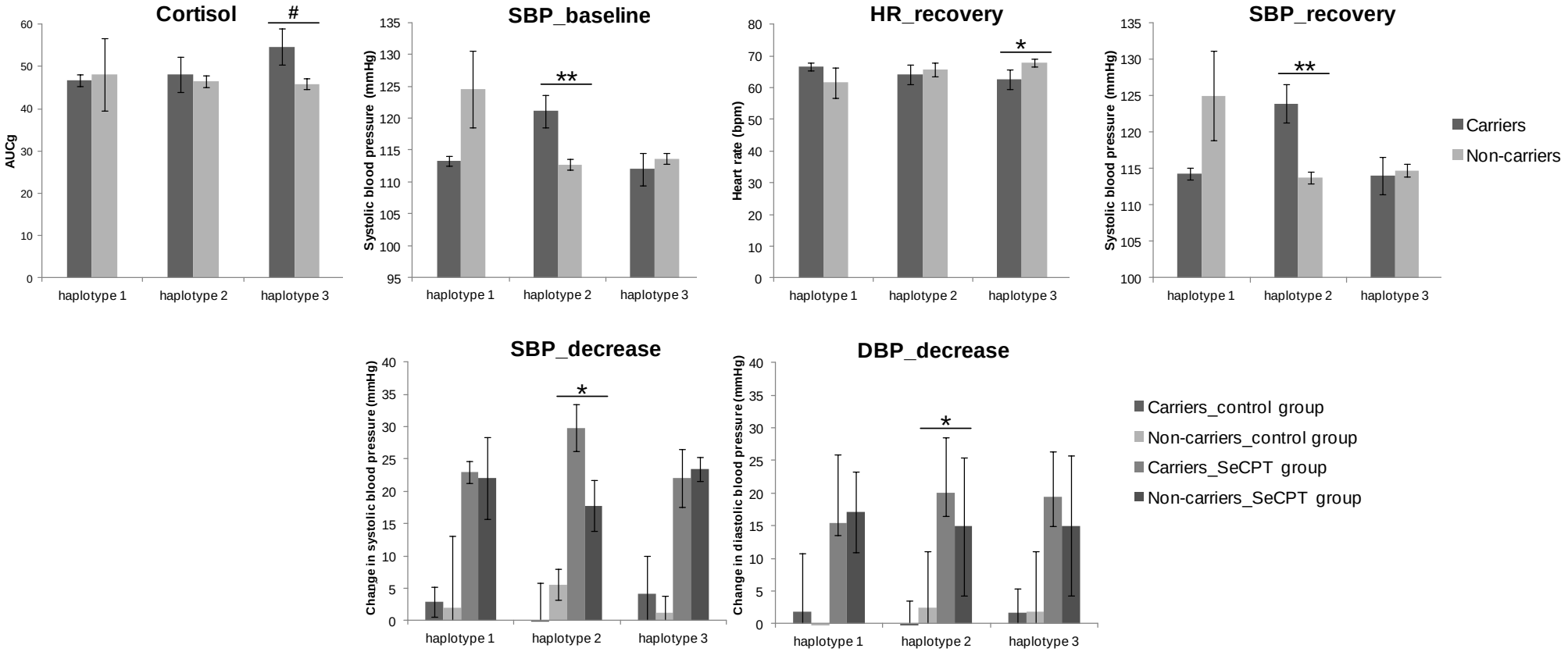


Figure 4.3. Mean values corresponding to ANOVA results: Asterisks indicate a significant main effect of haplotype (carriers, noncarriers) in the upper section or a significant interaction of a haplotype (carriers, noncarriers) and the socially evaluated cold pressure (SeCPT) manipulation in the lower section. Baseline data were considered as an average value of three measurements before the water task. Recovery was calculated using the three measurements after the water task. Decrease referred to the dependent variables from the peak to the recovery period. The results are represented as the mean ± SEM. (*$p < 0.05$; ** $p < 0.001$, #$p = 0.05$) Abbreviation: AUCg: Area under the curve with respect to ground; DBP: Diastolic blood pressure; Cortisol: post-awakening cortisol; HR: Heart rate; SBP: Systolic blood pressure.

Stress condition

Five repeated measures of cortisol levels during the SeCPT served as an indicator for the HPA axis reactivity. Cardiovascular data (HR, SBP, DBP) as well as AUCg and AUCi calculated from five cortisol measures were used as the dependent variable in separate between-participants ANOVAs with the two factors SeCPT group and haplotypes (Table 4.2). There was a significant main effect of haplotype 2 on the “SBP recovery”, with higher SBP for carriers [F (1, 212) = 13.429, p< 0.001] compared to non-carriers (mean 123.912 ± 2.64; mean 113.777 ± 0.825). Moreover, “SBP decrease” was influenced by a significant interaction of haplotype 2 and SeCPT group [F (1, 209) = 4.860, p = 0.029]. Carriers of haplotype 2 showed a higher “SBP decrease” in the SeCPT group whereas in the control group carriers of that haplotype had a smaller “SBP decrease”. Similar, “DBP decrease” was also influenced by a significant interaction of haplotype 2 and SeCPT group [F (1, 212) = 6.831, p = 0.010]. In addition, there was a significant main effect on the “HR recovery” with lower HR for haplotype 3 carriers [F (1, 212) = 13.429, p = 0.036] compared to non-carriers (62.641 ± 3.065; 67.831 ± 1.354).

Table 4.2: The association of haplotypes of the *CRH* promoter with the cortisol level, blood pressure and heart rate under basal and stress condition.

Genotypes and Haplotypes	haplotype 1 (N = 213)		haplotype 2 (N = 21)		haplotype 3 (N = 20)	
	main effect[a]	interaction[b]	main effect[a]	interaction[b]	main effect[a]	interaction[b]
Basel condition						
Cortisol level						
AUCg						
AUCi						
Blood pressure						
SBP_baseline						
DBP_baseline						
Heart rate						
bpm_basline						
Stress condition						
Cortisol level						
AUCg_SeCPT						
AUCi_SeCPT						
Blood pressure						
SBP_increase						
SBP_peak						
SBP_decrease						
SBP_recovery						
DBP_increase						
DBP_peak						
DBP_decrease						
DBP_recovery						
Heart rate						
bpm_increase						
bpm_peak						
bpm_decrease						
bpm_recovery						

> 0.05 | 0.01-0.05 | 0.001-0.009

Abbreviations: AUCg: area under the curve with respect to ground; AUCi: area under the curve with respect to increase; bpm: beats per minute; DBP: diastolic blood pressure, SBP: systolic blood pressure, SeCPT: socially evaluated cold pressure test; [a] haplotype as main effect; [b] interaction between haplotype and group; *p* values are provided according to a color code as indicated by the appended scale.

4.5 Discussion

Three *CRH* haplotypes constituted of the two SNPs rs5030875 and rs3176921 were investigated *in vitro* and *in vivo* regarding their possible influence on stress regulation. In the first part of the study, we characterized the haplotypes in two cell lines with respect to their baseline activity as well as their activation after stimulation with forskolin and hydrocortisone. Haplotype 2 (G-C), which contains the minor alleles of rs5030875 and rs3176921, showed the highest transcriptional activity in both cell lines under baseline conditions and after treatment with forskolin. The stimulation with hydrocortisone alone, however, increased the promoter activity of all three haplotypes in AtT-20 cells but not in U373MG. In our study, we choose hydrocortisone as it can, rather than dexamethasone, activate both types of glucocorticoid receptors. Further, tissue-specific actions of ligand-activated glucocorticoid receptors might take place on the *CRH* promoter in a cell-type-specific way (King & Nicholson, 2007). In addition and in contrast to other studies, our promoter construct contains not only the proximal 600 to 900 bp of the *CRH* promoter but also the distal promoter, which harbors more potential transcription factor binding sites that might indirectly enhance and inhibit the regulation via GCs (Vamvakopoulos & Chrousos, 1993). In the second part, we found that under baseline conditions carriers of haplotype 2 showed an effect on the SAM system, represented by higher blood pressure compared to non-carriers, but did not differ in cortisol levels. After stress exposure, haplotype 2 was associated with different blood pressure indices but again not with any HPA-related measure. In addition, we observed a trend for carriers of haplotype 3 to possess higher post-awakening cortisol compared to non-carriers.

In our study, we worked with AtT-20 cells, a widely used cell line in the studies focusing on CRH (King & Nicholson, 2007), and U373 cells as a human cell line to have two possible models for the CRH action. It is known that CRH expression is highly tissue specific, with highest expression levels found in placenta followed by brain, testis, and thyroid (Fagerberg et al., 2014). Interestingly, only one *CRH* promoter appears to be responsible for this tissue-specific regulation of CRH expression. For example, in pituitary tumor-derived AtT-20 cells

cAMP produces a high level of promoter activity through the cAMP response element (Jensen et al., 2009), and the caudal type homeobox protein response element (CDXARE), whereas in placental cells the *CRH* promoter had a low intrinsic basal activity and cAMP caused a modest increase in activity because CRE acts in isolation (King & Nicholson, 2007). Therefore, AtT-20 and U373MG cells might differ in their capacity to mediate signals to the *CRH* promoter, which could explain differences observed in our experiments. Functional consequences of the investigated haplotypes are supposed to vary in different cell types and seem to depend on specific TFs. These differences can be related to the actual TFs present, splice variants, cofactors, post-translational modifications, differences in activation pathways, or sequence variations. As such, our findings might inspire further research on the biological mechanisms and need additional evaluation.

Previously and similarly to our study, a significant increase in the promoter activity after stimulation with 8-Br-cAMP has been reported (Wagner et al., 2006). However and in contrast to our findings, haplotype 1 (A1B1) resulted in the highest luciferase activity after 36 hours. This difference might be attributed to the longer stimulation time and/or to the use of different stimulation reagents. Both, 8-Br-cAMP and forskolin, are related to cAMP activation but serve different molecular mechanisms. In our study, we used forskolin, an activator of the adenylate cyclase that increases intracellular levels of cAMP and its availability for different TFs. Moreover, a previous study suggested that the effects of forskolin on the AtT-20 cell may be due to mechanisms occurring in normal pituitary cells, too (Guild et al., 1986). In comparison, Wagner and colleagues treated cells with 8-Br-cAMP, a brominated derivate of cAMP, which is long-acting on the activation of cAMP-dependent protein kinases. Due to the stimulation of intracellular cAMP production and possible recruitment of specific cAMP-dependent TFs (Kapatos et al., 2000; Wagner et al., 2006), forskolin might lead to the slightly different activation profile observed in our settings.

We focused on two SNPs that were previously shown to impact mainly cAMP-dependent transcription factor binding. Rs3176921 changes the consensus sequence of the binding box of nuclear transcription factor Y (NF-Y) from CCAAT to CCACT, and therewith disrupts NF-Y

binding (Wagner et al., 2006) as well as significantly affects the expression of genes involved in complex pathways (Chassanidis et al., 2009; Chen et al., 2007; Dolfini et al., 2012). Further evidence is accumulating that alteration of the NF-Y structure is not the direct cause of any specific disease. Rather the efficiency of DNA-binding might play a role on the development of several pathological conditions, resulting either in altered proteins, or altered gene expression patterns (Dolfini et al., 2012). Further, rs5030875 is in a high linkage disequilibrium with the SNP rs5030876, which results in the exchange of a nucleotide within the binding site of the transcription factor activating transcription factor 6 (ATF6). ATF6 is a member of the human ARF/CREB (cAMP response element binding protein) family. ATF6 participates in two independent signaling pathways, both leading to transcriptional activation in the nucleus (Yoshida et al., 1998), interaction with cAMP responsive elements (Fawcett et al., 1999), and finally differences in the *CRH* promoter activity (Wagner et al., 2006).

The pivotal role of CRH in integrating various aspects of the stress response by regulating the HPA axis is well understood. In particular, stress-induced hypothalamic CRH results in the release of ACTH, which increases blood pressure secondary to the secretion of glucocorticoids from the adrenal cortex. In addition to these indirect effects, CRH also exerts direct effects on the cardiovascular system, which can be centrally and peripherally. Therefore, several pathways have been discussed (Yang et al., 2010). First, stress-induced hypothalamic CRH enhances contractility of vasculature and increases blood pressure and heart rate via β-endorphin-induced catecholamine release from the adrenal medulla. Second, central CRH modulates autonomic nervous system activity via the dorsal motor nucleus of the vagus, decreasing heart rate and increasing heart rate variability due to enhanced sympatho-vagal antagonism. Third, peripheral CRH mediates subsequent decrease in blood pressure and relaxation of vasculature, while direct action on CRHR2 in the heart enhanced myocyte contractility (Yang et al., 2010). Complementary to our *in vitro* findings, we found independent of the stress exposure higher blood pressure in carriers of haplotype 2 under baseline conditions. This might reflect the direct rather than the indirect effects of CRH on the cardiovascular system, probably due to a haplotype dependent increase of *CRH* expression.

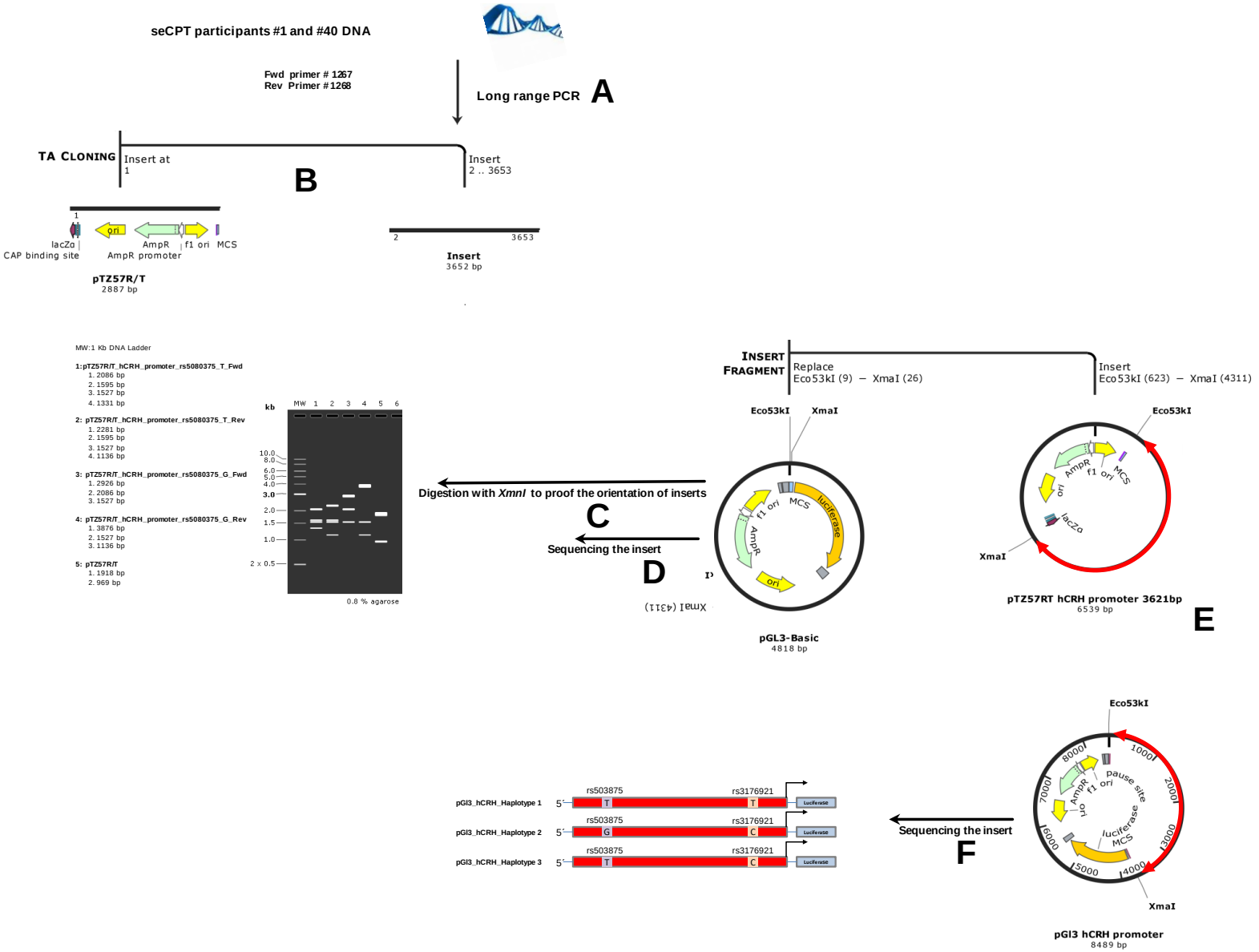
We suggest that more CRH binds to the CRH receptors but did not have a significant impact on cortisol levels in our cohort. In contrast, the observed trend for higher post-awakening cortisol (calculated as AUCg) and lower heart rate recovery of carriers of haplotype 3 indicates genetic impacts on the HPA that are unrelated to blood pressure. This might reflect a protective aspect of higher AUCg and only moderate changes under stress. Although the present findings do to not permit to explain the exact pathways of the observed effects, they demonstrate the putative importance of the examined SNPs in stress responses and susceptibility to situational and individual influences. We suggest considering and testing the various ways of CRH affecting the cardiovascular system (directly or indirectly) when trying to understand the association between stress responses and genetic variations.

For the interpretation of our study results, some limitations need to be taken into account. To control for false positive results of promoter activity only due to the inserted DNA sequences, the h*CRH* promoter region was also used in an antisense orientation. The promoter activity of our constructs was tested in two tumor cells derived from pituitary or glioblastom but not in peripheral cells. However, as central CRH signaling is involved in HPA axis regulation as well as direct regulation of cardiovascular processes, the investigation of those cells seemed a plausible choice. Even with a sample size of over 200 participants, the low-frequency (MAF 5-10 %) of many genetic variants are outside the reach of sufficient statistical power for association studies. Therefore, some challenges continue to exist in both the discovery and interpretation of findings from genetic association studies. Given the sample size of N = 218 in our study, the statistical power to detect a small-to-medium-size of $\eta_p^2 = 0.04$ was $1 - \beta = 0.76$ for analyses comparing three haplotype groups. Although this power can be considered as adequate for the present purpose of our investigation, the significance of the reported associations still has to be interpreted carefully because smaller effects might have remained undetected. In particular, a careful interpretation of the association results related to haplotype 2 and 3 is warranted due to the small sample size. In addition, to control for the relatively small number of donors, we aimed at a high homogeneity of the young, infrequent-smoking, undergraduate student population reducing confounding socioeconomic factors.

Hence, our results should not be generalized to other than a Caucasian undergraduate student population. For future studies, it might be necessary to increase the samples size and population diversity. Perhaps, the reported associations change with age or differ across ethnicities. If available, a focus on low-frequency alleles might be considered in particular.

4.6 Conclusion

The present study links the functional inspection *in vitro* and the genetic association of *CRH* promoter SNPs *in vivo* to physiological stress parameters. *In vitro* assays demonstrated differences in promoter activity depending on the haplotype. Haplotype 2 revealed the highest transcriptional activity under baseline conditions and after treatment with forskolin. The molecular mechanism underlying these differences might be affected by the SNPs that change DNA sequences and therewith important regulatory elements. *In vivo*, individuals with haplotype 2 had a higher dynamic response in systolic blood pressure under baseline and after stress exposure compared to non-carriers. Taken together, we conclude that these *CRH* haplotypes contribute to individual differences in the neuroendocrine response when coping with stress. In addition, our results suggest that direct and indirect effects of CRH differ regarding the individual susceptibility to situational stimuli and genetic variance.


seCPT participants #1 and #40 DNA
Fwd primer # 1267
Rev Primer # 1268
Long range PCR
A
TA CLONING
Insert at 1
Insert 2 .. 3653
B
lacZα
CAP binding site
ori
AmpR promoter
AmpR
f1 ori
MCS
pTZ57R/T
2887 bp
Insert
3652 bp
MW:1 Kb DNA Ladder
1: pTZ57R/T_hCRH_promoter_rs5080375_T_Fwd
1. 2086 bp
2. 1595 bp
3. 1527 bp
4. 1331 bp
2: pTZ57R/T_hCRH_promoter_rs5080375_T_Rev
1. 2281 bp
2. 1595 bp
3. 1527 bp
4. 1136 bp
3: pTZ57R/T_hCRH_promoter_rs5080375_G_Fwd
1. 2926 bp
2. 2086 bp
3. 1527 bp
4: pTZ57R/T_hCRH_promoter_rs5080375_G_Rev
1. 3876 bp
2. 1527 bp
3. 1136 bp
5: pTZ57R/T
1. 1918 bp
2. 969 bp
kb
MW 1 2 3 4 5 6
10.0
8.0
6.0
5.0
4.0
3.0
2.0
1.5
1.0
2 x 0.5
0.8 % agarose
Digestion with XmnI to proof the orientation of inserts
C
Sequencing the insert
D
XmaI (4311)
INSERT FRAGMENT
Replace Eco53kI (9) – XmaI (26)
Insert Eco53kI (623) – XmaI (4311)
Eco53kI
XmaI
f1 ori
MCS
luciferase
AmpR
ori
pGL3-Basic
4818 bp
lacZα
pTZ57RT hCRH promoter 3621bp
6539 bp
E
pGl3_hCRH_Haplotype 1
pGl3_hCRH_Haplotype 2
pGl3_hCRH_Haplotype 3
rs503875
rs3176921
5'
T
G
C
Luciferase
Sequencing the insert
F
pause site
pGl3 hCRH promoter
8489 bp

SM-Figure 4.1: Cloning strategy for *CRH* promoter constructs.
(A) Long-range 3651 bp PCR product (primers see SM-Table1) was sequenced and the amplified fragments were confirmed to be identical with the genomic *CRH* sequence. (B) The respective long-range 3651 PCR product was previously sub-cloned into the TA Cloning vector, pTZ57R/T (InsTAclone PCR Cloning Kit, Thermo Scientific™ K1213). (C) To identify the *CRH* promoter construct (~6.5 kb), the pTZ57R/T−h*CRH* promoter plasmid was additionally digested with *Xmn*I to identify the orientation of inserts. (D) The resulting right-hand fragment (~3.7 kb) was purified after agarose gel electrophoresis using a DNA Extraction Kit (Invitek, Berlin, Germany). The resulting right-orientation pTZ57R/T−h*CRH* promoter plasmid was confirmed by DNA sequencing using DNA Sequencing Kit, Beckmann Sequencing. (E) This pTZ57R/T−h*CRH* promoter plasmid was digested with restriction enzymes *Xma*I and *Eco*53kI and ligated at a ratio of 3:1 (insert to vector) to similarly digested and purified pGL3−Basic vector (Ligase). The luciferase construct (~8.5 kb) was used to transform XL1 blue competent cells. Miniprep DNA (Qiagen, Hilden, Germany) was fractionated on an agarose gel to screen for pGl3_h*CRH* promoter incorporation. The identity of the candidate clone was further verified by colony PCR (primer see SM-Table1). (F) The integrity of all reporter constructs was confirmed by DNA sequencing using DNA Sequencing Kit (Beckman Coulter).

SM-Table 4.1: PCR primers for construction of h*CRH* reporter plasmid and subsequent sequencing.

Applications	Primer name	Primer (5'--3')
Long-range PCR	hCRH_promoter Fwd	ACCCTAGGCATTTCTGAAGGC
	hCRH_promoter Rev	CTCTTGACAGCTCGATTGCG
Sequencing	M13	GTAAAACGACGGCCAGT
	M13	CAGGAAACAGCTATGAC
	Sequencing Primer_1 Fwd	ATCAGGTGCCACAGACATGA
	Sequencing Primer_1 Rev	TCATGTCTGTGGCACCTGAT
	Sequencing Primer_2 Fwd	GGTTGTTCCCATGCTGTTCT
	Sequencing Primer_2 Rev	AGAACAGCATGGGAACAACC
	Sequencing Primer_3 Fwd	GAAGAACCTCCCAGACCTCC
	Sequencing Primer_3 Rev	GGAGGTCTGGGAGGTTCTTC
	Sequencing Primer_4 Fwd	GCTACAAAGGAAGCAGCAGAA
	Sequencing Primer_4 Rev	TTCTGCTGCTTCCTTTGTAGC
	Sequencing Primer_5 Fwd	ACTGGCCGCCTCTCTCTTTA
	Sequencing Primer_5 Rev	TAAAGAGAGAGGCGGCCAGT
	Sequencing Primer_6 Fwd	TGGTCAGGGAGGTTAGGAGA
	Sequencing Primer_6 Rev	TCTCCTAACCTCCCTGACCA
	pGl13_RVprimer3 Fwd	CTAGCAAAATAGGCTGTCCC
	pGl13_GLprimer2 Rev	CTTTATGTTTTTGGCGTCTTCCA
Colony PCR	CRH rs3176951 Fwd	TTGAAATATGCATTGCATCATTTGT
	CRH rs3176951 Rev	ACACAAACTGAGGTGAAAAGATGAA

SM-Table 4.2: PCR primers and conditions for the detection of *CRH* polymorphisms

Gene	SNP	Sequence[a]	Tm[b] (ºC)	Mg^{2+} (mM)	Primers (µM)
CRH	rs3176921	Fwd: 5'-ttgaaatatgcattgcatcatttgt -3' Rev: 5'-atatccctttgctagagacagag -3'	66	2.5	0.4
	rs5030875	Fwd: 5'-tgaaggtacaaggtgatacaagtgacaa-3' Rev: 5'- acacaaactgaggtgaaaagatgaa -3'	60	2.5	0.1

[a]Fwd, forward or sense primer ; Rev, reverse or antisense primer. Primer with restriction enzyme recognition sites (underlined) used for amplification of the promoter fragments.

[b]Tm, annealing temperature in PCR.

SM-Table 4.3: Overview of genotype and haplotype frequencies in the sample investigated.

SNP/Name	Genotype/Haplotype	N	Control group	SeCPT group
rs5030875	G/G	5	1	4
	T/G	32	11	21
	T/T	181	57	124
rs3176921	T/C	21	6	15
	T/T	197	63	134
	C/C	0	0	0
haplotype1	T-T	213	68	145
		5	1	4
haplotype2	G-C	21	8	13
		197	61	136
haplotype3	T-C	20	7	13
		198	62	136

SM-Table 4.4: Descriptive data of two single nucleotide polymorphisms of the *CRH* in 218 participants using Haploview

SNPs	position	obsHET	predHET	HWpval	% Geno	MAF	Alleles
rs5030875	8:66181831	0.105	0.1	1.0	100.0	0.053	T : G
rs3176921	8:66179144	0.162	0.185	0.1481	100.0	0.103	T : C

Abbreviations: **obsHET**: observed heterozygosity; position: chromosomal location; **predHET**: predicted heterozygosity; **HWpval**: p value for Hardy-Weinberg-Equilibrium; **% Geno**: Genotyping frequency; **MAF**: minor allele frequency;

4.7 Acknowledgement

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Chapter 5 General discussion

The main objective of the experiments described in this thesis was to understand the role of HPA-related genetic factors in stress responses. Genetic factors represent one of the components causing individual variations in physiological stress parameters. Despite intensive research on the HPA-related genes by various association studies in stress-response investigations over the last two decades, the current knowledge on mechanisms underlying these associations is still fragmentary. Therefore, continued efforts are necessary to explore these associations on different genetic levels. Following this idea, this thesis examined HPA-related genes on three different levels. The first level was the influences of several genotypes and haplotypes of genes, which are involved in stress regulation by the HPA axis, examined physiologically by baseline HPA axis activity and psychologically by self-perceived stress in a healthy cohort. The second level was the effect of *GR* variants (genotypes and haplotypes) and promoter methylation level, which were involved in stress regulation in response to the SeCPT and affected indicators of the SAM system as well as HPA axis activity in the same healthy cohort. The third level comprised the characterization of *CRH* promoter haplotypes in an *in vitro* study and association of the CRH promoter haplotypes with parameters of the HPA axis and the SAM system in an *in vivo* study.

An interpretation of the present findings raises fundamental questions: What are the directions of the links reflected by the observed significant associations? Which models are available for fitting these findings? It is worth mentioning that, when trying to analyze and explain the results of genetic association studies, simple linear thinking should be avoided. The human body is one excellent and highly complex system which involves several compliant sub-systems, such as the stress-response system focused on in this thesis. It is necessary to use a systematic approach for analyzing the present findings. Richmond (1987) coined the term “systems thinking”. Systems are made up of three parts: elements, interconnections, and a function or a purpose. In

the stress-response systems, the elements are different organs, such as the brain, gland, and the blood vessels and so on. The interconnections would be the physical flow of different hormones. The function of the stress response system is to allow the human body to keep the balance. Additionally, Wright and Meadows (2008) identify flow and stock as parts making up systems. A stock serves as a setpoint for every system. Flows are the actions impacting a system. They include inflow (input) and outflow (response). When these three parts keep in balance, the system can run normally. When a system displays a behavior that is consistent over time, it is highly likely that there is a mechanism working to control and create that behavior. This mechanism works through a feedback loop. There are two types of feedback loops responsible for producing dynamic behavior, positive feedback loops and negative feedback loops. Positive feedback loops reinforce the stock whereas negative feedback loops are balancing. For instance, in the stress-response system, GCs form an important negative feedback loop.

The two concepts of homeostasis and allostasis are related to the setting of a setpoint. Whereas homeostasis refers to the ways a body keeps stability, allostasis comprises the dynamic processes that achieve this balance. Allostasis means stability by changing. Homeostasis means stability by staying the same (Sterling, 2012). Thus, according to homeostasis, physiological regulation has the function to match internal parameters to setpoints by negative feedback in response to registered errors. Correspondingly, physicians typically assume defective internal mechanisms when parameters deviate from setpoints and design therapies to re-establish normal values. In contrast, allostasis does not consider a deviating parameter to reflect a failure but understands it as a reaction to a certain prediction. As a consequence, the allostasis model focuses on enhancing high-level restoration of predictive fluctuation instead of manipulating low-level mechanisms (Sterling, 2014).

As mentioned previously, following system thinking to analyze the present results, it is impossible to consider all the factors involved in the system. However, thinking “simple” by focusing on selected elements and connections is the first step to explore the complexity of the system. Some models might help to understand interconnecting factors. Mostly, four possible types have been considered by recent genetic association studies (Gutierrez-Arcelus et al., 2013), based on the idea of quantitative trait loci (QTL) that are sections of DNA loci that are associated with variation in a quantitative trait (phenotype). These are the expression Quantitative Trait Loci (eQTLs), the methylation Quantitative Trait Loci (mQTLs), the expression Quantitative Trait Methylation (eQTMs) and the *expression methylation* Quantitative Trait Loci (emQTLs, Figure 5.2). In this thesis, the loci are focused on SNPs of the HPA-axis related genes. A quantitative methylation (QTM) is DNA methylation which correlates with variation in the quantitative trait. Standard eQTLs or eQTMs analysis involves a direct association test between makers of genetic variations or DNA methylation with gene expression levels. Moreover, DNA methylation at specific loci can be influenced by sequence variations, such that individual genotypes at a given locus may result in different patterns of DNA methylation due to allele-specific methylation. These sites are called methylation Quantitative Trait Loci (mQTLs). There are also interactions between the specific loci and DNA methylation which also could correlate with the quantitative trait (expression methylation Quantitative Trait Loci; emQTLs). Gutierrez et al. (2013) distinguished potential causative models. Under the independent model (INDEP), a SNP influences DNA methylation and gene expression independently. Under the SNP-methylation-expression model (SME), a SNP influences gene expression through methylation. Under the SNP-expression-methylation model (SEM), a SNP influences methylation through gene expression. They also refer to another "Interaction" (SNP_Methylation_SNP*Methylation-expression) model, the interaction between SNP and methylation, which then affects gene expression (Figure 5.1).

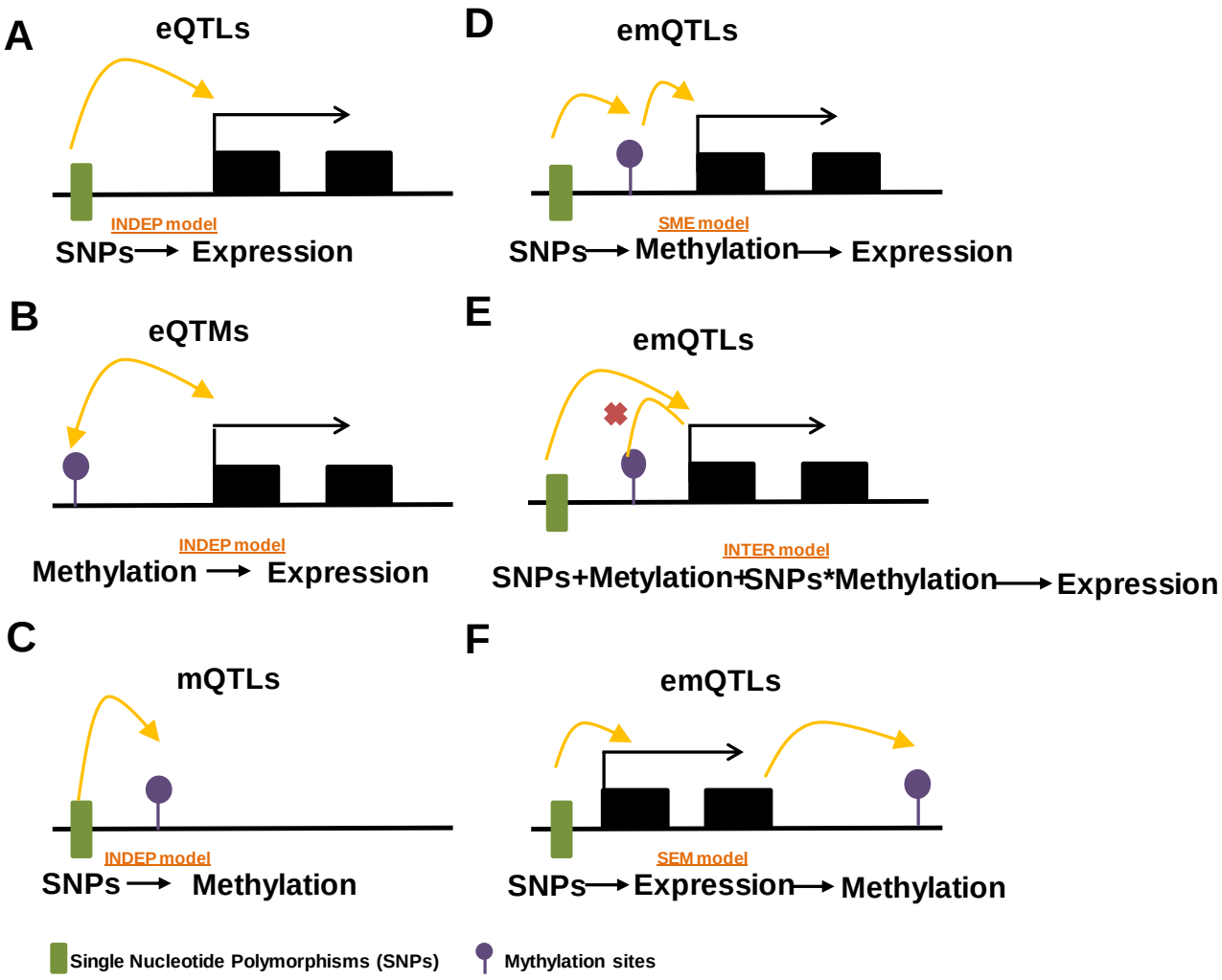


Figure 5.1 Schematic representations of four different molecular-mechanism types in genetic associations and six different causative models. **A**: Expression Quantitative Trait Loci (eQTLs) under independent model (INDEP); **B**: Expression Quantitative Trait Methylation (eQTMs) under INDEP model; **C:** Methylation Quantitative Trait Loci (mQTLs) under INDEP model; **D**: Expression methylation Quantitative Trait Loci (emQTLs) under SNP-methylation-expression (SME) model; **E**: Expression methylation Quantitative Trait Loci (emQTLs) under interaction (INTER) model; **F**: Expression methylation Quantitative Trait Loci (emQTLs) under SNP-expression-methylation (SEM) model. Black boxes represent exons and orange arrows depict associations between SNPs, methylation, and gene expression. Figure adapted after Gutierrez et al. (2013).

In this thesis, the indicators of the HPA axis and the SAM system also can be considered as "endophenotypes", in a similar manner as gene expression factors in the four causative models discussed above. Following this idea, there are three different types available for interpreting the function of the stress-response related genes in the HPA axis and the SAM system. In an adaptation of the terms introduced above, these are the stress-response Quantitative Trait Loci (rQTLs), the stress-response Quantitative Trait Methylation (rQTMs) and the stress-response

methylation Quantitative Trait Loci (rmQTLs). Their corresponding causative models are shown in Figure 5.2.

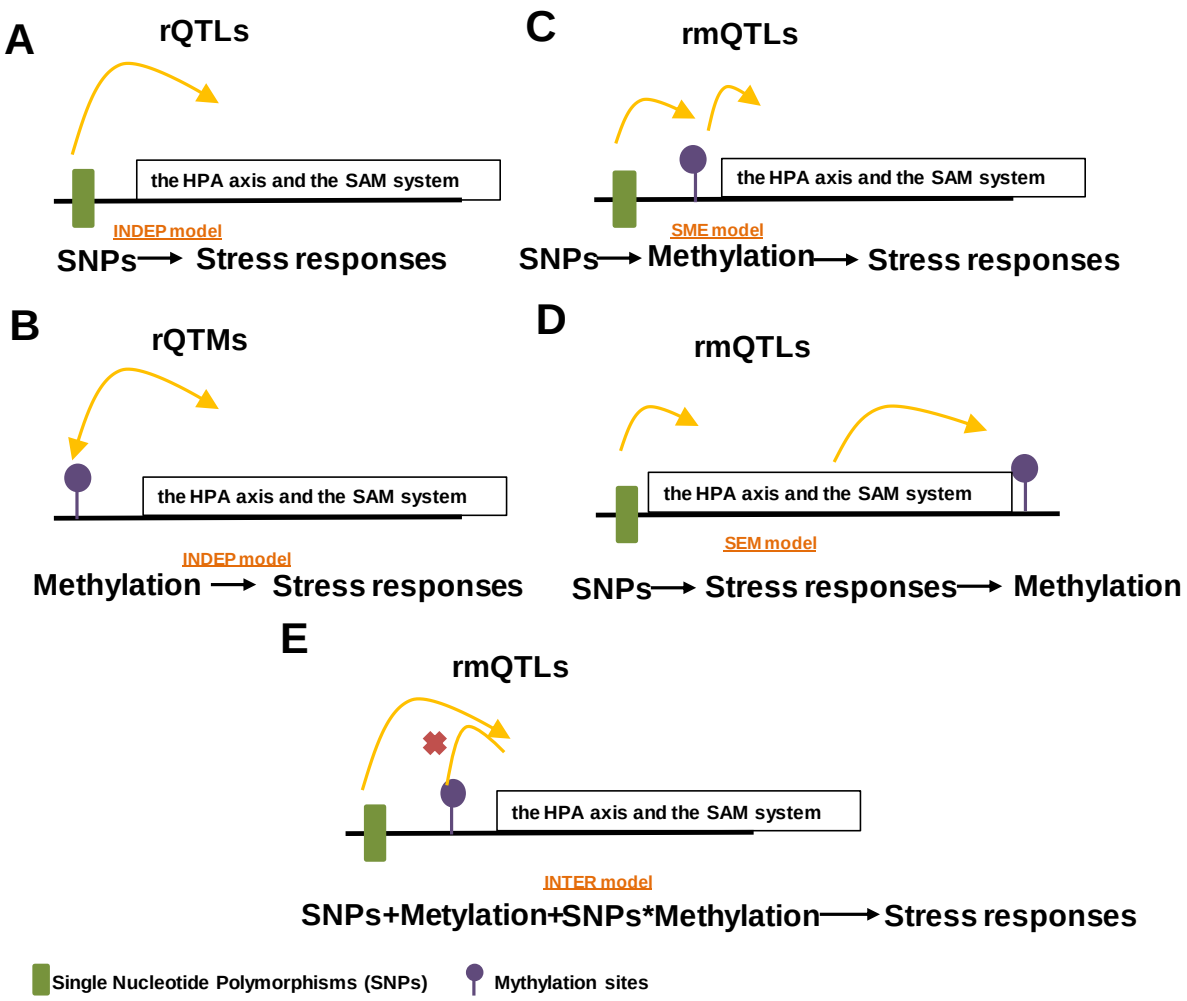
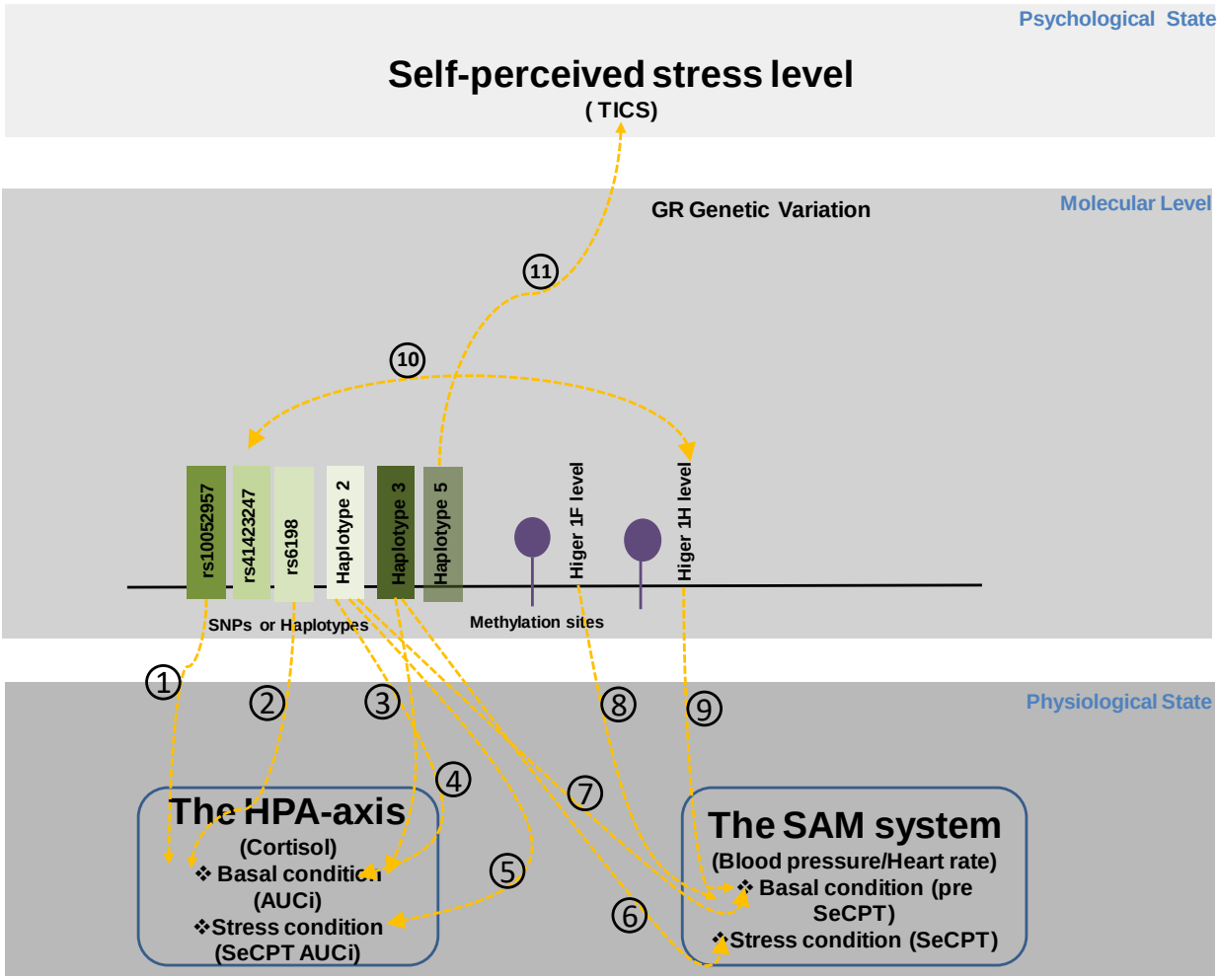


Figure 5.2 Schematic representations of three different types of stress responses in the HPA axis and the SAM system in genetic associations and their corresponding five different causative models. **A**: Stress-responses Quantitative Trait Loci (rQTLs) under INDEP (independent) model; **B**: Stress-responses Quantitative Trait Methylation (rQTMs) under INDEP (independent) model; **C**: Methylation Quantitative Trait Loci (mQTLs) under SMR (SNP- methylation- stress-responses) model; **D**: Stress-responses methylation Quantitative Trait Loci (rmQTLs) under SRM (SNP- stress-responses -methylation) model; **E**: Stress-responses methylation Quantitative Trait Loci (rmQTLs) under INTER (interaction) model. Orange arrows depict associations between SNPs, methylation, and stress responses in the HPA axis and the SAM system.

Moreover, as reflected in the present results, compared to other stress-response related genes, *GR* and *CRH* might show their central links in a triangular association as indicators of both the HPA axis and the SAM system. Therefore, the following paragraph will focus on discussing potential processes behind the observed significant associations in these analysis models.

a



b

NO.	Significant Results	Models	Causative models
①	Woman homozygous for the minor T allele of the *Tth*III*I* SNP showed higher AUCi than women homozygous for the minor allele	rQTLs	INDEP
②	Male minor G allele carriers of the 9 beta SNP had higher AUCi compared to men homozygous for the major A allele	rQTLs	INDEP
③	The AUCi of male carriers of haplotype 3（minor allele of *Tth*III*I*, NR3C1-1 and 9 beta) was higher than the AUCi of male non-carriers	rQTLs	INDEP
④	Male carriers of haplotype 2 (minor allele of *Bcl*I) displayed a significantly lower AUCi compared to male non-carriers	rQTLs	INDEP
⑤	GR haplotype 2 carriers had a higher cortisol response to the seCPT	rQTLs	INDEP
⑥	Haplotype 3 carriers had a stronger heart rate, which decreased post stress	rQTLs	INDEP
⑦	Haplotype 2 had a higher cortisol response to the seCPT and higher heart rate and higher blood pressure independently of the experimental group	rQTLs	INDEP
⑧	Increased GR 1F methylation levels were significantly associated with decreased baseline blood pressure	rQTMs	INDEP
⑨	Increased GR 1H methylation levels were significantly associated with decreased baseline blood pressure	rQTMs	INDEP
⑩	The *Bcl*I minor allele correlated with high sum promoter 1H methylation levels	mQTLs	INDEP
⑪	Male carriers of GR haplotype 5 had higher scores on the TICS scales “work overload” and “pressure to succeed” compared to female carriers	rQTLs	INDEP

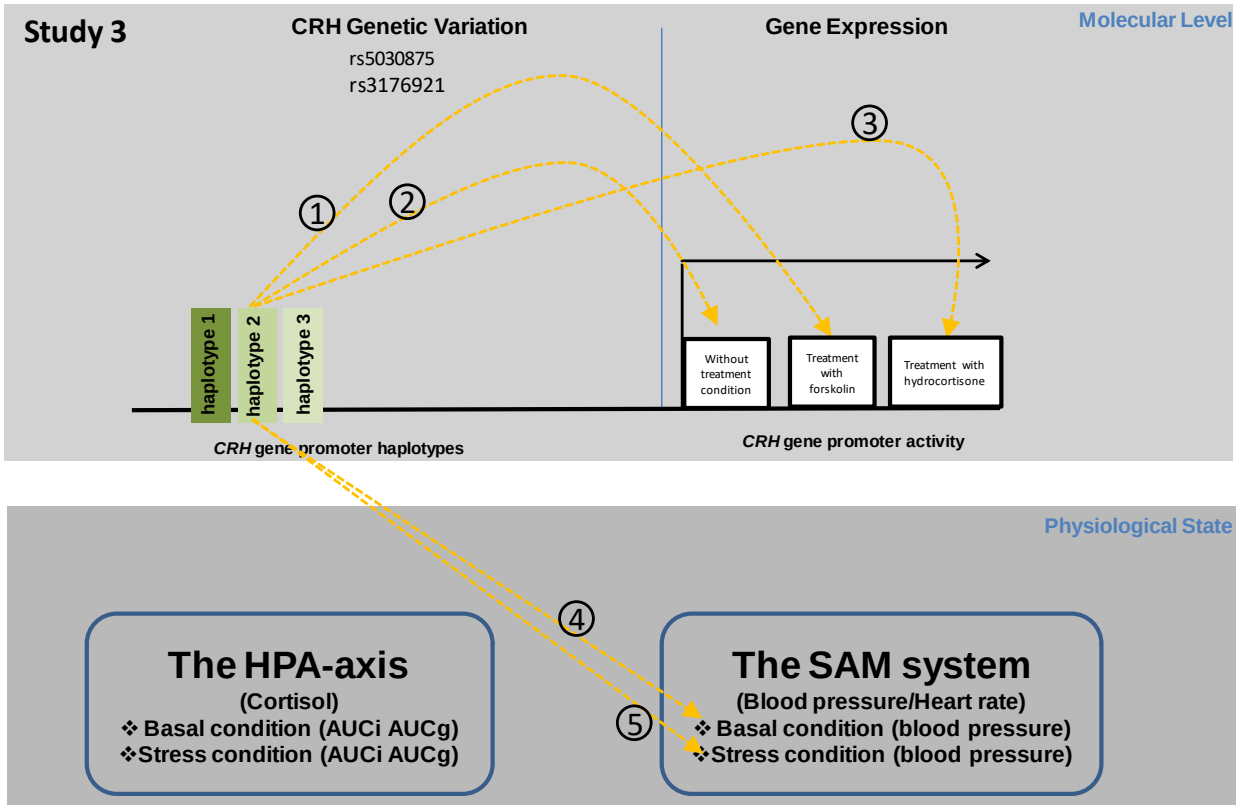
Figure 5.3 Significant associations of the *GR* SNPs and haplotypes in study 1 and study 2 linked to causative models. **a**: A schematic overview of the significant results of the *GR*. Number 1 to 11 present individual significant results. **b**: Individual results linked to types and causative models.

As Figure 5.3 showed, ① to ⑦ and ⑪ belong to rQTLs and independent causative models (model A in Figure 5.2). ⑧ and ⑨ belong to rQTMs and independent causative models (model B in Figure 5.2). ⑩ is a mQTLs type and independent causative model (model C in Figure 5.2). When combining the results of ⑨ and ⑩, it matches the rmQTLs and SMR causative models (model C in Figure 5.2). Model D could not be found in these studies, because the methylation level was not tested again after the stress response. Model E could not be tested because of a need for higher sample size, due to the low frequency of the respective SNPs.

Figure 5.4 shows the findings of Study 3: ① to ③ belong to eQTLs and independent causative models (model A in Figure 5.1). ④ and ⑤ belong to rQTLs and independent causative models (model B in Figure 5.2). C, D, E and F models cannot be tested because the methylation level of CRH gene was not examined. Still, this study suggests links between gene expression and stress response. Thus, a new study seems promising detecting more factors regarding gene expressions, methylation levels, and stress response at baseline levels as well as reactivity levels in both the HPA axis and the SAM system. It would be also interesting to increase the sample numbers to detect possible interactions between SNPs and methylation level impacting gene expression and stress response.

Whereas comparatively little is known about molecular mechanisms involved in mQTLs, a variety of mechanisms has been identified regarding eQTLs and eQTMs. Molecular mechanisms that can be affected by eQTLs comprise 1) promoter activity (e.g. *CRH* promoter haplotype 2 in study 3), 2) transcription efficiency (e.g. a positive influence of *GR TthIII*I on *GR*-1B mRNA expression; a negative influence of *GR* SNP NR3C1-1 (rs10482605) on transcriptional activity), 3) gene splicing (e.g. an influence of *GR* 9beta on the ratio of GRalpha to GRbeta), 4) mRNA stability and 5) translation efficacy (e.g. a negative influence of *GR Bcl*I on expression of GRalpha protein isoform).

a



b

NO.	Significant Results	Types	Causative models
①	Promoter haplotype 2 of CRH showed the highest transcriptional activity in both cell lines under baseline conditions	eQTLs	INDEP
②	Promoter haplotype 2 of CRH showed the highest transcriptional activity in both cell lines after treatment with forskolin	eQTLs	INDEP
③	Promoter haplotype 2 of CRH showed the increased transcriptional activity in AtT-20 cells but not in U373MG cells after treatment with hydrocortisone	eQTLs	INDEP
④	Under baseline conditions, carriers of haplotype 2 showed an effect on the SAM system, represented by higher blood pressure compared to non-carriers, but did not differ in cortisol levels	rQTLs	INDEP
⑤	After stress exposure, haplotype 2 was associated with different blood pressure indices but again not with any HPA-related measure	rQTLs	INDEP

Figure 5.4 The significant associations of the *CRH* in study 3 linked to types and causative models. **a** A schematic overview of the significant results of the *CRH*. Number 1 to 5 presents individual significant results. **b** Individual results linked to types and causative models.

Molecular mechanisms that can be affected by eQTMs comprise 1) gene silencing, 2) gene transcription efficiency, 3) alternative intragenic promoter activity, 4) tissue-specific regulatory elements, 5) binding of transcription factors and 6) splicing (alternative). The present studies did not test eQTMs, therefore, no exemplary results are listed here.

Most of the results presented in this thesis belong to an independent model. It is necessary to consider the typically low frequency regarding minor allele frequencies of the examined polymorphisms. Biologically informed multilocus profiles seem to be a promising way of extending the utility of this research (Bogdan, Hyde & Harri, 2013). Such profiles represent the combined effect of several polymorphic loci. Concerning the HPA axis, it might be interesting to focus on low-frequency alleles in informative biological genes in larger samples in combination with those multilocus analyses. Building on the findings in this thesis, it is possible to compile individual genetic profile scores reflecting the total number of variants that have each been shown to be associated with the HPA axis and the SAM system. For example, across all loci, relatively 'high' HPA reactivity genotypes could be assigned a score of 1, 'medium' genotypes a score of 0.5, and 'low' HPA reactivity genotypes a score of 0. An individual profile score then results from totalling scores at each locus.

Moreover, as mentioned previously, the allostasis model is an alternative to homeostasis that suggests dynamic regulatory processes (Sterling & Eyer, 1988). It proposes that the goal of efficient regulation is not constancy, but instead fitness under natural selection. Following this approach, Juster and colleagues (2010) recently tried to identify biomarkers that are believed to contribute to an allostatic load (AL). The AL model assumes multi-systemic interactions between primary mediators and effects on stress response and suggests measures to detect the individual AL level. This approach aims at calculating AL scores and summarizes levels of physiological activity across the HPA axis and the SAM system. The AL index is a measure that serves as a quantified tool for checking stress levels. The allostasis model is sometimes considered the hallmark of health. The original AL index included data from 10 physical or physiological measures. These were the four primary mediators urinary epinephrine, norepinephrine, cortisol, and dehydroepiandrosterone sulfate (DHEA-S), as well as the six secondary outcomes: total cholesterol (TC), high-density lipoprotein (HDL), waist-to-hip ratio (WHR), glycosylated hemoglobin (HbA1c), systolic blood pressure, and diastolic blood pressure

(Mauss et al., 2015). According to the original description, these data are transformed into a summary score by assessing the distribution of each value within the screened sample and assigning those within the highest risk quartile (lowest quartile of DHEA-S and HDL-values) the value "1". These binary indicators, all of which are equally weighted, are then added. Accordingly, the range was between 0 and 10, with lower values reflecting better adaptation to stress and higher values reflecting higher physiological strain (Mauss et al., 2015). There are several possible ways to interpret and develop this index further by using the findings of this thesis. For example, the multiple biomarkers of the HPA axis and the SAM system together with more anthropometric data (such as WHR or BMI) could be supplemented by genetic factors for calculating a dynamic allostatic load index score before and after stress responses. Furthermore, the measure of the allostatic load index score used most often is a simple count score and, thus, does not consider interactions of individual factors. It might be more appropriate when a formula also considering interactions between factors are used. The significant results from the present studies could provide us with some important hints for setting up this algorithm. For example, high blood pressure might only increase the allostatic load in combination with a major allele of a certain SNP but not with the minor allele of that SNP if an interaction effect between the SNP and blood pressure is taken into account. Another way of adapting the calculation could be to also include weights of the individual components that reflect their relative impact.

Correspondingly, the significant results of genetic association studies in this thesis can give hints on some interconnections within the stress-response systems. No direct linear causal links exist (such as a change in A leads to a change in B) but more complex networks (such as a change in A simultaneously changes C, D, E… finally resulting in a change in B). Other influences need consideration, such as environments, cognitive process, or the human gut microbiome and so on.

Further directions for future studies are suggested by the three perspectives that guided this dissertation: influences of SNPs, influences of methylation, and combining *in vivo* with *in vitro* studies regarding SNPs. The studies of this thesis are among the first to link epigenetic

modifications of the *GR* promoter, receptor genotype, and physiological measures of the stress response, as well as to detect *CRH* promotor haplotypes *in vivo* and *in vitro*. *CRH* and *GR* are suggested as two essential genes to focus on for more intense research in stress-response studies. Especially regarding the *CRH* gene, it is possible to link promoter haplotypes, the promoter activity *in vitro*, epigenetic modifications of the CRH promoter and physiological measures of both the HPA axis and the SAM system. There are several possible directions for further investigations. For example, one direction could be to focus on links between *CRH* and the microbiome in healthy subjects as well as obese subjects. The microbiome has been found to be related to various parameters of both the HPA axis and the SAM system. In addition, knowing involved candidate genes is considered in current trends in individual medicine regarding anti-stress treatment. A promising way could be to combine big-data analysis with calculating allostatic load index scores and individual multilocus genetic profile.

Chapter 6 Summary

Genetic factors represent one of the components causing individual variation in physiological stress parameters. The physiological stress system includes the HPA axis and SAM system. The primary aim of this thesis was to test how genetic factors influence both of these stress systems. Five genes involved in the functioning of the HPA axis regarding stress responses are examined in this thesis. They are: corticotropin-releasing hormone (*CRH*), the glucocorticoid receptor (*GR*), the mineralocorticoid receptor (*MR*), the 5-hydroxytryptamine-transporter-linked polymorphic region (5-*HTTLPR*) in the serotonin transporter (5-*HTT*) and the brain-derived neurotrophic factor (*BDNF*) gene. Two-hundred-thirty-two healthy participants were genotyped and the influence of genetic factors on physiological parameters such as post-awakening cortisol and blood pressure as well as on stress reactivity measured after socially evaluated cold pressor test (SeCPT) were investigated. Three studies tested the HPA-related genes each on three different levels. The first level was the influences of genotypes and haplotypes of these five genes on physiological as well as psychological stress indicators (**Chapter 2**). The second level was the effects of *GR* variants (genotypes and haplotypes) and promoter methylation levels on both the SAM system and the HPA axis stress reactivity (**Chapter 3**). The third level comprised the characterization of *CRH* promoter haplotypes in an *in-vitro* study and the association of the *CRH* promoter with stress indicators *in vivo* (**Chapter 4**).

The main results of the three studies are: Study 1 (**Chapter 2**) showed influences of several *GR* SNPs and haplotypes on the cortisol awakening response and on self-reported stress that partially were moderated by gender. Study 2 (**Chapter 3**) showed associations of *GR* promoter methylation levels as well as *GR* haplotypes with blood pressure and heart rate. In addition, one *GR* SNP (*Bcl*I) was associated with *GR* promoter methylation. Study 3 (**Chapter 4**) showed a link between a *CRH* promoter haplotype with transcriptional activity *in vitro*. *In vivo*, *CRH* haplotypes were associated with blood pressure.

Compared to other stress-response related genes examined in this thesis (*MR*, 5-*HTT* and *BDNF*), *GR* SNPs and *CRH* promoter SNPs might possess central links in triangular associations, influencing both the HPA axis activity and the SAM system. Overall, the observed associations contribute to individual susceptibility to stress which is controlled by an exquisite mix of genetic and epigenetic factors.

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Erklärung

nach § 9, Abs. 1 der Promotionsordnung des Fachbereichs I der Universität Trier vom 13.11.2008.

Hiermit versichere ich, dass ich die vorliegende Arbeit selber verfasst und keine außer den angegebenen Hilfsmitteln und Referenzen benutzt habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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