A Psychobiological Perspective on the Association of Common Glucocorticoid Receptor Gene Polymorphisms with Hypothalamus-Pituitary-Adrenal Axis Regulation

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Table of Contents

Abbreviations

A	adenine
ACTH	adrenocorticotropic hormone
AF-1	activating function 1
ANOVA	analysis of variance
AP1	activator protein 1
AUC	area under the curve
AVP	arginine-vasopressin
Bp	basepair
BMI	body mass index
BNST	bed nucleus of the stria terminalis
C	cytosine
CAR	cortisol awakening response
CBG	corticosteroid binding globulin
CRH	corticotrophin-releasing hormone
DBD	DNA binding domain
Dex	Dexamethasone
DNA	deoxyribonucleic acid
DST	Dexamethasone Suppression Test
ELISA	Enzyme Linked Immunosorbent Assay
fMRI	functional magnetic resonance imaging
G	guanine
GABA	γ-aminobutyric acid
GC	glucocorticoid
GLM	general linear model
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIP1	glucocorticoid receptor interacting protein-1
h	hours
HPA	hypothalamus-pituitary-adrenal
Hsp	heat shock protein
IFN-γ	interferon-γ
IL	interleukin
K	lysine

Kb	kilobase
LBD	ligand binding domain
LPS	lipopolysaccharid
mRNA	messenger RNA
min	minutes
MR	mineralocorticoid receptor
ms	milliseconds
nGRE	negative glucocorticoid response element
NGFI-A	nerve growth factor-induced protein A
NFκB	nuclear factor κ B
OC	oral contraceptives
PET	positron emission tomography
POMC	pro-opiomelanocortin
PTSD	post traumatic stress disorder
PVN	paraventricular nuclei
R	arginine
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAGE	serial analysis of gene expression
SEM	standard error of the mean
SNP	single nucleotide Polymorphism
SRp30c	serine-arginine-rich protein 30c
Stat5	signal transducer and activator of transcription 5
T	thymine
TAD	transactivating domain
TNF-α	tumor necrosis factor
TSST	Trier Social Stress Test
UTR	untranslated region
WHR	waist-to-hip ratio
5-HT	serotonin
5-HTT	serotonin transporter
11β-HSD	11β-hydroxysteroid dehydrogenase

Index of Figures and Tables

Figures

Tables

CHAPTER 1

Introduction and Outline

1.1 Introduction

Mental disorders constitute a major burden for society. In 2005, the European Brain Council announced the results of a pan-European project to analyze the prevalence and cost of all mental disorders in Europe (Andlin-Sobocki et al 2005). Across 28 European countries with a total population of 466 million, 127 million people or 27% are affected by at least one brain disease and mental disorders are associated with immense total costs of over 290 billion Euros per year (Wittchen and Jacobi 2005). Mental disorders are complex in their etiology and numerous factors are known to interact in the course of their development. In broad terms it can be stated, that, as proposed by the diathesis-stress model, a genetic vulnerability or predisposition (the diathesis) interacts with the environment and life events (stress) to trigger behaviors or psychological disorders (Zubin and Spring 1977). Indeed, the relationship between mental disorders and stressful life events is well established, both in epidemiological and clinical samples (Paykel 2003), and virtually all psychiatric disorders are closely linked with stress (Young 2004). The question remains, however, why some people who are exposed to an environmental pathogen, e.g. psychological stress, develop mental disorders while others do not. The stress response has evolved as a highly adaptive reaction that ensures survival when an organism is confronted with physiological or psychological challenge. Thus, we are confronted with the dilemma that the same responses, which are adaptive under acute stress conditions, can ultimately promote or sustain disease processes when occurring chronically. Stress physiology has attracted enormous research interest and almost hundred years of investigation have deepened our understanding of the physiological processes, down to the molecular level, elicited under stress. One important stress responsive system is the hypothalamus-pituitary-adrenal (HPA) axis, a hierarchical hormonal system, which mediates the endocrine stress response. This system is under tight self-regulating control through negative feedback mechanisms. The glucocorticoid receptor (GR), scrutinized in this thesis, plays a crucial role in these processes. Interestingly, almost all mental disorders have been shown to be associated with alterations in the HPA axis (McEwen 1998) and these dysregulations are associated with, if not caused by, altered GR functioning. The exact mechanisms how a failure to cope with stress can result in molecular changes and consequently precipitate a disease state are just beginning to be understood. The vulnerable phenotype model proposes that responses to stressors depend on genetic predisposition and are

modulated by the history of the individual, particularly during early life or even prenatally (de Kloet et al 2005).

How can a genetic predisposition be identified? Until the era of molecular genetics, a genetic predisposition was observed when the occurrence of particular disorders tended to run in families. Furthermore, twin studies allowed estimating the heritability, i.e. the part of variance of a trait explained by genetic factors, of personality traits and disease. Thus, the general influence of genetic factors could be estimated, however, these approaches do not allow the identification of the involved, or predisposing, genetic loci. In 2004, following the publication of a rough draft in 2001 (Lander et al 2001), the effort to sequence the human genome was completed and revolutionized medical genetics (International Human Genome Sequencing Consortium, 2004). Not only did the Human Genome Project determine the exact sequence of the human genome and identify the approximately 25,000 genes, more importantly, it provided information about the differences in the genetic makeup of individuals. The human genome has about 10 million polymorphisms, defined as genetic variants in which the minor forms have a prevalence of at least 1% in the population (Goldstein and Cavalleri 2005). The most common type of variants in our genome are single nucleotide polymorphisms (SNPs), the exchange of one base pair through another. Small changes to the genome such as SNPs can exert considerable effects on cellular and tissue level, which can ultimately affect the entire physiology of an organism. SNPs do not invariably *cause* but *predispose* us to common disease, in combination with other genetic variants and the environment we are exposed to. Thus, the main use of a human SNP map will be in dissecting the contributions of individual genes to diseases that have a complex, multigene basis (Chakravarti 2001). Genomic variations are thought to underlie differences in our susceptibility to, or protection from all kinds of disease. In the realm of neuroscience, this knowledge „...promises to provide unprecedented opportunities to explore the genetic basis of individual differences in complex behaviors and vulnerability to neuropsychiatric illness“ (Hariri and Weinberger 2003).

In order to identify individuals at risk for the development of psychiatric disease following stressful events, the underlying genetic architecture of stress-responsive system has to be thoroughly characterized. Experimental work presented in this thesis is intended to contribute to a further understanding of the influence of genetic factors on the functioning of the HPA axis. The aim is to describe the relative contributions of genetic variation of the GR, a key-regulator of this stress-responsive

system, on the regulation of the HPA axis under various stimulation procedures. The characterization of HPA axis response phenotypes in individuals carrying different GR genotypes can be a first step in the identification of individuals who are vulnerable to or protected against the development of stress-related disorders.

1.2 Outline

A consistent feature of HPA axis activity is considerable individual variation in response dispositions (Mason 1968). A number of factors accounting for the observed variability have been identified, including, among others, sex (Kudielka and Kirschbaum 2005), chronic stress (Schulz et al 1998) exposition to early trauma (Heim et al 1998) or maternal prenatal stress (Wadhwa 2005). The question to what extend HPA regulation is influenced by genetic factors has not been studied extensively. Although substantial heritability measures for HPA axis responses have been documented in twin studies (Federenko et al 2004; Wüst et al 2005), the contribution of variation in single genes implicated in HPA axis regulation has not been thoroughly investigated. Genetic variations of the GR are likely to constitute a factor in the observed variability of HPA responses. A large number of polymorphisms of the glucocorticoid receptor gene have been identified, however, the number of variants relevant for the explanation of variance in the general population is likely to be small. Four SNPs of the GR have been studied more or less extensively and associations with measures of body composition, metabolic parameters and indices of GC sensitivity could be revealed (see Chapter 2). Given this evidence, functional relevance of these variants for GC sensitivity seems obvious. Thus, the aim of this thesis is to investigate the influence of all common GR gene polymorphisms with known functionality or previously reported associations and sufficient prevalence in the population (ER22/23EK, N363S, *Bcl*I, 9beta) on HPA axis activity following a psychosocial stressor, sensitivity to exogenous glucocorticoid administration and on working memory performance.

The general introduction in the present **Chapter 1** is intended to explain the rationale of the research strategy underlying the line of work presented in this thesis. **Chapter 2** presents the theoretical background on the topic and is intended to briefly define the term stress and highlight the brain processes involved in the regulation of our organism's stress-sensitive systems. One focus will be on the role of corticosteroid receptors in stress physiology and it will be addressed, how the same

responses that allow adaptation to a stressor can eventually promote disease processes. Emphasize will be put on the role of altered GR signaling in these processes and the molecular mechanism of GR functioning and GC signaling will be described in detail. In **Chapter 3**, HPA axis responses following a psychosocial stress protocol in the different GR genotype groups are presented. **Chapter 4** investigates GR genotype groups with regard to differences in glucocorticoid sensitivity in different tissues, i.e. peripheral leukocytes, subdermal blood vessel and pituitary. In **Chapter 5**, the impact of GR gene polymorphisms on working memory performance under cortisol and placebo administration is scrutinized. **Chapter 6** is intended to highlight availability of glucocorticoids as another key element affecting GC signaling. For this purpose, the effect of corticosteroid binding globulin (CBG), a key regulator of glucocorticoid availability, on HPA axis responses to pharmacological and psychological stimulation is presented. **Chapter 7** provides a general discussion of the findings followed by an outlook in **Chapter 8** where future research directions are delineated.

Chapters 3-6 are written so that they can be read separately, making a certain amount of redundancy unavoidable. These chapters represent manuscript drafts that will be submitted for publication to different journals. Experimental work presented was conducted in collaboration with Prof. Dr. Hellhammer, Dr. Stefan Wüst and Sonja Entringer from the University of Trier and with Elisabeth van Rossum and Jan Willem Koper from Erasmus Medical Center, Rotterdam. Since not all subjects were subjected to every experiment conducted, the number of investigated subjects shows slight variation in the different chapters.

References

Andlin-Sobocki P, Jonsson B, Wittchen HU, Olesen J (2005): Cost of disorders of the brain in Europe. *Eur J Neurol* 12 Suppl 1:1-27.

Chakravarti A (2001): To a future of genetic medicine. *Nature* 409:822-823.

de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475.

Federenko IS, Nagamine M, Hellhammer DH, Wadhwa PD, Wüst S (2004): The heritability of hypothalamus pituitary adrenal axis responses to psychosocial stress is context dependent. *J Clin Endocrinol Metab* 89:6244-6250.

Goldstein DB, Cavalleri GL (2005): Genomics: understanding human diversity. *Nature* 437:1241-1242.

Hariri AR, Weinberger DR (2003): Imaging genomics. *Br Med Bull* 65:259-270.

Heim C, Graham YP, Heit SC, Bonsall R, Miller AH, Nemeroff CB (1998): Increased sensitivity of the hypothalamic-pituitary-adrenal axis to psychosocial stress in adult survivors of childhood abuse. *Soc Neurosci Abst* 28:201.212.

International Human Genome Sequencing Consortium (2004): Finishing the euchromatic sequence of the human genome. *Nature* 431:931-945.

Kudielka BM, Kirschbaum C (2005): Sex differences in HPA axis responses to stress: a review. *Biol Psychol* 69:113-132.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al (2001): Initial sequencing and analysis of the human genome. *Nature* 409:860-921.

Mason JW (1968): A review of psychoendocrine research on the pituitary-adrenal cortical system. *Psychosomatic Medicine* 30:576-607.

McEwen BS (1998): Stress, adaptation, and disease. Allostasis and allostatic load. *Ann N Y Acad Sci* 840:33-44.

Paykel ES (2003): Life events and affective disorders. *Acta Psychiatr Scand Suppl*:61-66.

Schulz P, Kirschbaum C, Pruessner J, Hellhammer DH (1998): Increased free cortisol secretion after awakening in chronically stressed individuals due to work overload. *Stress Med* 14:91-97.

Wadhwa PD (2005): Psychoneuroendocrine processes in human pregnancy influence fetal development and health. *Psychoneuroendocrinology* 30:724-743.

Wittchen HU, Jacobi F (2005): Size and burden of mental disorders in Europe--a critical review and appraisal of 27 studies. *Eur Neuropsychopharmacol* 15:357-376.

Wüst S, Federenko IS, van Rossum EF, Koper JW, Hellhammer DH (2005): Habituation of cortisol responses to repeated psychosocial stress-further characterization and impact of genetic factors. *Psychoneuroendocrinology* 30:199-211.

Young AH (2004): Cortisol in mood disorders. *Stress* 7:205-208.

Zubin J, Spring B (1977): Vulnerability--a new view of schizophrenia. *J Abnorm Psychol* 86:103-126.

CHAPTER 2

Theoretical Background

2.1 Stress and the Brain: From Adaptation to Disease

2.1.1 Defining Stress

The term stress was originally taken from the dynamics of physics to describe „the mutual actions which take place across any section of a body to which a system of forces is applied“ (see Levine 2003). Walter Cannon, who first used the stress term in a biological context, defined stress in terms of the stimulus required to elicit adrenomedullary responses (Cannon 1914; Cannon 1915; Cannon 1932). The other pioneer in stress research, Hans Selye, who was also responsible for popularizing the concept in the biomedical community (Sapolsky 1994), defined stress in terms of responses of the endocrine, autonomic and immune system (Selye 1936; Selye 1956). Since then, numerous attempts to define stress have been undertaken, each emphasizing different components. Levine and Ursin (1991) pointed out that the stress concept is a composite and multidimensional concept with interacting subclasses. The three main subclasses can be identified as the stress stimulus (the input), the processing system and the stress response (the output). The stress system affects many physiological processes and “may function as a common alarm and drive system, whenever there is a real or apparent challenge to the self-regulating systems of the organism”.

Whereas Lazarus & Folkman (1984) put emphasize on the transactional element of stress, defining psychological stress as “a particular relationship between the person and the environment that is appraised by the person as taxing or exceeding his or her resources and endangering his or her well-being“, the definition of Chrousos and Gold (1992) has the concept of homeostasis at the core. Homeostasis is defined as a dynamic and harmonious equilibrium that is constantly challenged by intrinsic or extrinsic disturbing forces. Living organisms survive by maintaining this state and this is achieved by adaptational responses consisting of physical and mental reactions that are activated to counteract the effects of stressors in order to reestablish homeostasis. In biomedical research, the concept of stress as a state of threatened homeostasis is the most prominent one and stress “often refers to situations in which adrenal glucocorticoids (GCs) and catecholamines are elevated because of an experience” (McEwen 2000). Taken together, stress can be viewed as an adaptive response of an organism in response to threats of physiological or psychological well-being.

2.1.2 Acute Stress

The effects of stress become manifest in behavior, subjective experience, cognitive function and physiology (Steptoe 2000). There is a surge in arousal, focused attention, vigilance, alertness and cognitive processing. Peripherally, physiological and behavioral responses are triggered aimed at reinstating homeostasis, reflected in activation of the sympathetic nervous system and a rise in GC concentration through activation of the hypothalamus-pituitary-adrenal (HPA) axis. Activation of the HPA axis plays a crucial role in adaptation to homeostatic challenge and GCs are presumed to restore homeostasis following disruption. GCs act at virtually all levels of the body through binding to the glucocorticoid receptor (GR; see below). The end effects of GCs include, among others, energy mobilization, suppression of several immune functions, potentiation of sympathetic nervous system-mediated vasoconstriction and suppression of reproductive function (Sapolsky 2000). Another important function of GCs is the exertion of negative feedback at multiple brain sites to restrain the stress response and adequately control GC secretion (Chrousos and Gold 1992; Jacobson and Sapolsky 1991). These processes are coordinated by distinct stress-responsive systems in the brain and will be described below.

2.1.3 Stress Neurocircuitry

The fact that the triggered responses are both essential for survival and are remarkably consistent in their presentation has led to the suggestion that a discrete neuronal system has evolved for the coordination of the adaptive responses observed under stress (Chrousos and Gold 1992). The two principal components governing the stress response are the corticotropin releasing hormone (CRH) and locus coeruleus-norepinephrine system. In this context, the central mechanisms controlling the CRH system and thereby hypothalamo-pituitary-adrenocortical responsiveness will be highlighted.

The CRH neurons of the hypothalamic paraventricular nucleus (PVN) integrate excitatory and inhibitory inputs into a net secretory signal at the pituitary gland. Release of CRH and the co-expressed neuropeptide vasopressin (AVP) are essential for coordinating the stress response and for governing HPA axis activity. They trigger the release of ACTH from the pituitary, which results in secretion of GCs from the adrenals. The HPA axis has two modes of operation. One is the regulation of the diurnal rhythm of GC secretion and the other is the control of GC secretion following stress. Herman et al. (2003) hypothesize two distinct realms of stress activation.

Stimuli triggering ´reactive´ responses represent genuine homeostatic challenges recognized by somatic or visceral sensory pathways. These stressors would include pain, humoral homeostatic signals (e.g. changes in glucose or insulin levels) or humoral inflammatory signals. These inputs are mediated via direct innervations to the PVN from regions known to receive first- or second-order inputs from somatic nociceptors, visceral afferents or humoral sensory pathways and can therefore elicit rapid and reflexive activation of the HPA axis (see Herman et al 2003). Important for understanding physiological reactions to psychological or psychosocial stress is the fact that activation of the HPA axis can also occur in the absence of physiological challenge. These reactions are termed ´anticipatory´ responses and are centrally generated to mount a GC response in anticipation, rather than in reaction to, homeostatic disruption. Anticipatory responses can be elicited either by classically or contextually conditioned stimuli, i.e. memory programs, or innate species-specific predispositions. These innate programs include the recognition of predators or illuminated spaces for rodents, and also in humans, social challenges and unfamiliar environments or situations. In 1968 John Mason noted: „Psychological influences are among the most potent natural stimuli known to affect pituitary-adrenal cortical activity“ (Mason 1968). Situations characterized by novelty, uncontrollability and unpredictability, perception of threat and ego-involvement are known to reliably elicit HPA axis responses. Anticipatory responses are under control of limbic brain regions, which serve as the interface between the incoming sensory information and the appraisal process. Limbic regions known to influence the stress response include the hippocampus, nuclei of the amygdala, the lateral septum and the medial prefrontal cortex. However, none of these regions send direct projections to the PVN. Modulation of PVN activity is achieved through interactions with ´reactive´ stress circuits in brainstem, hypothalamic regions and regions of the bed nucleus of the stria terminalis (BNST) that directly innervate the PVN. Thus, limbic input is superimposed onto brainstem and hypothalamic stress effectors and a hierarchical system is formed capable of mediating both reactive and anticipatory stress responses.

2.1.4 Dynamics of the Stress Response: Role of Corticosteroid Receptors

Two modes of operation of the stress system have been suggested (reviewed by De Kloet et al. 2005). The system responsible for the initiation of the stress response, the fast mode, involves the above described CRH system, which drives the sympathetic and behavioral ´fight or flight´ response and activates the HPA axis. The

other slower mode terminates the stress response and thus promotes adaptation and recovery. Glucocorticoids operate in both modes through a dual receptor system, which consists of the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Both receptors bind cortisol in humans, albeit with considerable differences in affinity (De Kloet et al 1998; Reul and De Kloet 1985). As the affinity of the MR for cortisol is about tenfold higher than that of the GR, MR activation is maintained even under basal condition whereas the GR becomes activated during stress- and circadian-induced increases in GC concentration (Reul et al 2000). Based on these findings, different roles in HPA axis regulation were suggested for the two corticosteroid receptors: the MR, being occupied to about 80% under basal conditions, was thought to mediate the tonic inhibitory control on HPA axis activity, whereas GR mediate the negative feedback of elevated GC levels (De Kloet and Reul 1987). However, more recent studies indicate that the MR system is not a static system merely playing a cofactor role but rather represents a dynamic system responding to changing requirements, which participates in adaptive mechanisms in the brain evoked by stress. Findings supporting this view were presented, for instance, by Gesing et al. (2001) who report a transient increase in MR density following psychological stress. Furthermore, Cole et al. (2000) reported that administration of MR antagonist, but not GR antagonist, completely blocked habituation of adrenocortical reaction to repeated immobilization stress, supporting the view of a more dynamic role of the MR in the stress response. The notion that the MR is implicated in the appraisal process and the onset of the stress response (de Kloet et al 2005) is supported by findings demonstrating that corticosterone in the rat rapidly and reversibly changes hippocampal signaling through membrane-located MR (Karst et al 2005).

The GR, which becomes activated only by large amounts of GCs, terminates the stress response via the exertion of negative feedback at level of the pituitary, the PVN and at hippocampal sites (Herman et al 2003; Jacobson and Sapolsky 1991; Sapolsky et al 2000). Feedback mechanisms involve genomic DNA binding-dependent and -independent actions as well as rapid nongenomic actions (see section 2.2.1 for details). In summary, GCs in the brain act through two types of corticosteroid receptors allowing differential actions over the time course of the stress response. The MR is mostly responsible for the maintenance of the stress-related neural circuits, whereas the GR is important for the normalization of homeostasis.

2.1.5 Molecular and Cellular Changes

The dual role of the corticosteroid receptor system is also reflected in the concomitant molecular and cellular changes induced by activated MR and GR. As these receptors function as transcriptional regulators (see section 2.2.1), the initial step that leads to their ultimate physiological effects is the influence on expression patterns of responsive genes. Datson et al. (2001), using serial analysis of gene expression (SAGE), generated comprehensive expression profiles of rat hippocampus under different corticosterone conditions that mimic differential MR and GR occupation. This approach allows discriminating between MR- and GR-dependent transcriptional effects and revealed altered expression of over 200 genes. The majority of corticosterone responsive genes were regulated either by activated MR or GR and only about one-third of the genes were responsive to both types of receptor. Morsink et al. (2006) extended this approach and assessed transcriptional changes in a broader time window by generating a time curve of GR-mediated gene expression changes. Different waves of gene expression could be observed: at one hour after GR activation, responsive genes were exclusively down-regulated, shifting to both up- and down-regulation after three hours; five hours following activation, the response was almost back to baseline levels. Genes coordinated by GCs underlie aspects of cellular metabolism, protein synthesis, signal transduction and synaptic transmission. Altered transcription of genes involved in controlling the properties of ion channels, ionotropic receptors, G-protein-coupled receptors and ion pumps have been observed (Datson et al 2001; Morsink et al 2006). This leads to changes in the conductance of the plasma membrane and results in long lasting effects on neural transmission.

2.1.6 From Adaptation to Disease: Role of Altered GR Signaling

Alterations of the HPA axis have been associated with the development of a large number of psychiatric disorders (Young 2004). These observations reflect the dilemma, that the same responses that permit survival during acute stress can ultimately promote disease when occurring chronically. However, as not all individuals fall sick under conditions of chronic stress, research is directed towards elucidating the basis for such differences. There is considerable variation in HPA response disposition (Mason 1968) and it has been demonstrated that stress reactivity in adulthood can be modulated by genetic background (Degen et al 2004; Feldker et al 2003; Korte et al 2005; Landgraf and Wigger 2003; Veenema et al

2003; Wüst et al 2004), prenatal factors (Entringer et al in prep; Seckl and Meaney 2004; Wüst et al 2005), postnatal mother-offspring interaction (Francis et al 1999; Levine 1957; Liu et al 1997; Meaney et al 1988) and traumatic experience (Heim et al 2002; Kendler et al 2000). Adverse experience in early life in combination with the genetic background can produce a vulnerable phenotype (De Kloet et al 2005). Sensitization of limbic circuits responding to acute stressors can persistently alter HPA axis reactivity, which renders an individual more susceptible to develop stress-related disorders. Dysregulations of the HPA axis have been observed in various psychiatric illnesses including major depression (Holsboer 2000; Pariante 2003; Pariante and Miller 2001; Pariante et al 1995), bipolar disorder (Daban et al 2005; Rybakowski and Twardowska 1999), schizophrenia (Ryan et al 2004), anxiety disorders (Abelson and Curtis 1996) and post traumatic stress disorder (PTSD; Yehuda 1997). Both hyperactivity and hyper-reactivity of the HPA axis and impairments in negative feedback suppression (as observed in major depression) as well as increased negative feedback inhibition and relative hypoactivity of the HPA axis (as observed in PTSD, chronic fatigue symptomatology and burn-out; Heim et al 2000; Raison and Miller 2003) have been documented. Abnormal responses to neuroendocrine challenge tests such as the Dexamethasone suppression test or the combined Dexamethasone suppression / CRH stimulation test indicate that GR-mediated HPA regulation is impaired in these disorders. Further evidence pointing towards a central role of GR signaling in the development of psychopathology is the observed correlation of HPA abnormalities with changes in GR expression in psychiatric patients. For instance, reduced GR mRNA levels in the hippocampus and the frontal cortex of patients suffering from unipolar depression and schizophrenia have been observed (Perlman et al 2004; Webster et al 2002) and enhanced GR sensitivity associated with enhanced negative feedback inhibition of the HPA axis was revealed in patients with PTSD (Yehuda 2002; Yehuda et al 2004; Yehuda et al 2002). The development of psychiatric disorders is correlated with numerous other neurochemical abnormalities including changes in neurotransmitters and neuropeptides, so it is difficult to dissociate the primary causes from the secondary consequences of these diseases (Howell and Muglia 2006). In order to investigate the specific role of brain GR in regulation of the HPA axis and emotional behaviors, various transgenic murine models have been generated. Genetically altered GR lines include complete GR knockout (Cole et al 1995; Cole et al 1999; Cole et al 2001; Finotto et al 1999; Oitzl et al 2001), the Gr^{NesCre} line (Tronche et al 1999) with central

nervous specific GR knockout, FBGRKO (Boyle et al 2005) with forebrain-specific disruption of the GR, $GR^{dim/dim}$ (Reichardt et al 1998a) preventing DNA binding of the GR, GRov (Wei et al 2004), a mouse line overexpressing GR in the forebrain, $GR^{+/-}$ (Ridder et al 2005), a mouse line heterozygous for a GR null allele and mice globally overexpressing GR by a yeast artificial chromosome (YGR; Ridder et al 2005). Complete knockout of the GR is not compatible with life; however, mouse lines with more specific disruptions of the GR remain viable and display characteristic alterations in HPA axis regulation and emotional behavior, summarized in Table 2.1.

GR line	HPA regulation	Behavior
Gr^{NesCre}	Increased basal plasma CORT levels Reduced basal ACTH levels CORT response following stress unchanged Increased CRH expression in PVN	Reduced anxiety-like behavior Reduced cocaine-induced behavioral sensitization
FBGRKO	Increased basal plasma CORT levels Increased basal plasma ACTH levels Increased CORT and ACTH response following stress No suppression following Dex Increased AVP expression in PVN	Increased depression-related behaviors Increase in anhedonia Increased locomotor activity in stressful situations
$GR^{dim/dim}$	Increased plasma CORT levels Normal plasma ACTH Normal CRH expression in PVN Increased pituitary POMC expression	Normal anxiety-like behavior Impairment in spatial memory
GRov	No difference in circadian release of CORT and ACTH Normal CRH expression in PVN Increased CRH expression in rostral CeA	Increased anxiety-like behavior Increased despair-like behavior
$GR^{+/-}$	Increased CORT response following restraint stress and Dex/CRH challenge	No differences in depression or anxiety related behaviors Increased helplessness behavior after stress
YGR	Decreased CORT response following restraint stress and Dex/CRH challenge	Decreased helplessness behavior after stress

Table 2.1: *Endocrine and behavioral phenotype of GR transgenic mice*

Taken together, these results provide support for the notion that adequate regulation of GR signaling is critical for mental health. In the mouse models described above, a primary defect in central GR signaling results in altered HPA axis regulation and

changes in emotionally relevant behavior. These models help to better understand the pathways involved in the development of psychiatric disorders and to discern the relative role of altered GR signaling. Certainly, these specific mutations do not occur in humans. Nevertheless, these results support the notion that polymorphisms in the GR, which can impact on the efficacy of GC signaling and therefore profoundly influence the downstream biology of peripheral and central GC responsive systems, could lead to increased or decreased susceptibility to disease.

2.2 The Glucocorticoid Receptor

The Glucocorticoid Receptor (GR) is a member of the nuclear receptor superfamily, which, among others, includes the receptors for thyroid hormones, retinoids and vitamin D. Members of this superfamily are thought to be evolutionary derived from a common ancestor (Bridgham et al 2006; Laudet 1997). They exhibit a similar modular structure (Aranda and Pascual 2001) and the existence of a general molecular mechanism by which different receptors elicit their responses has been demonstrated. Nuclear receptors act as ligand-inducible transcription factors by directly interacting with DNA response elements, as well as by molecular cross-talk with other signaling pathways (Reichardt and Schütz 1998). The GR mediates the majority of the effects of cortisol, which is the most important glucocorticoid in humans. The physiological response and sensitivity to cortisol is known to vary among individuals, tissues and cell types (Breslin and Vedeckis 1998; Ebrecht et al 2000; Yudt and Cidlowski 2002). These differences in GC responses and efficacy of glucocorticoid signaling in general are largely determined by characteristics of the GR. In addition to GR isoforms resulting from alternative splicing (section 2.2.4) and translation initiation (section 2.2.5), polymorphisms of the GR gene (section 2.2.7) can considerably impact on GR functioning.

2.2.1 Molecular Mechanisms of Glucocorticoid Action

Genomic Actions

In the absence of ligand, the GR resides in the cytoplasm as part of a multi-protein complex. This complex consists of heat-shock proteins (Hsp), including Hsp 90, and several other chaperone proteins. Ligand binding induces conformational changes in the receptor, dissociation from the protein-complex, and nuclear translocation. GR exerts its effects via regulating the transcriptional activity of hormonal responsive genes or via protein-protein interactions. In the direct regulation of gene transcription,

the GR acts as a homodimer by binding to DNA motifs known as GC responsive elements (GRE, see Figure 2.1).

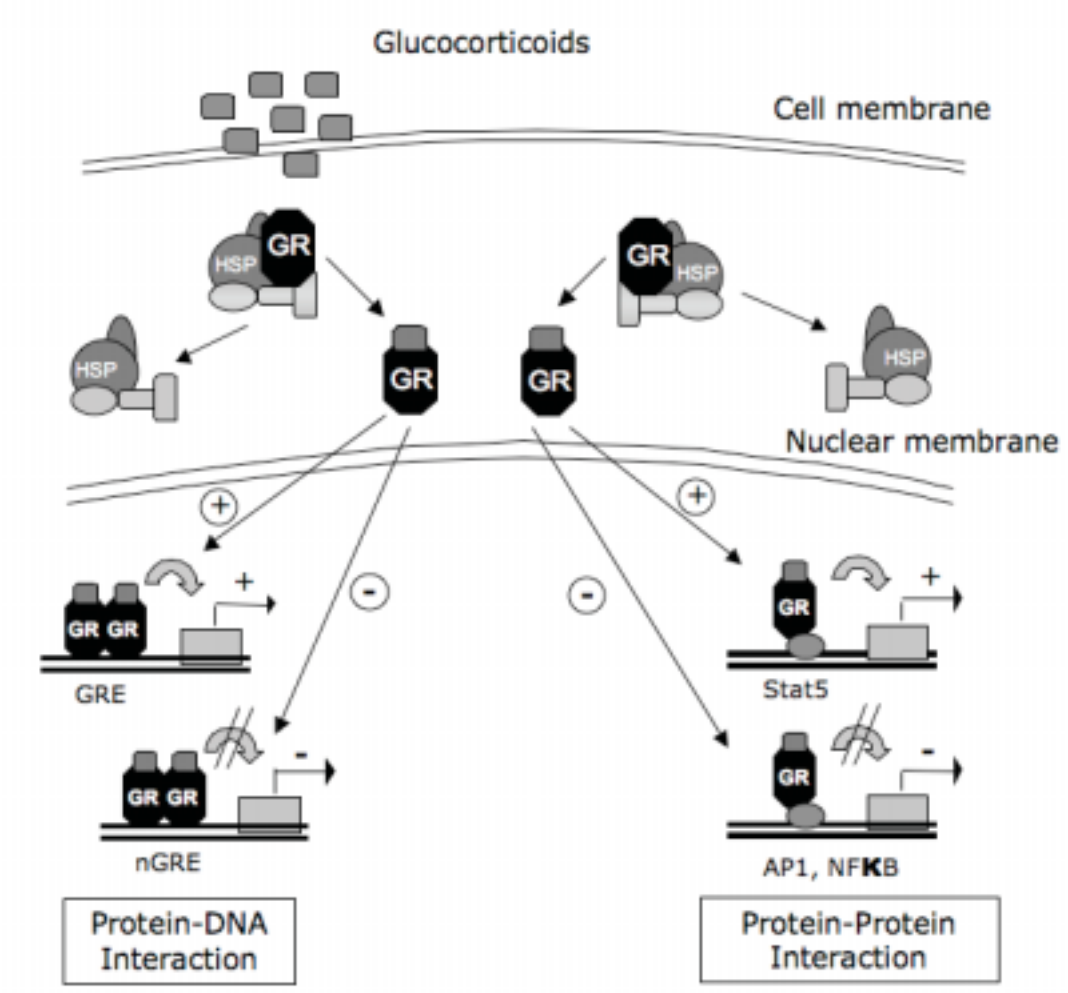

Figure 2.1: *Genomic actions of the GR. After entering the cell by passive diffusion, GCs bind to GR, which then dissociates from an Hsp90 containing protein complex and translocates to the nucleus. Binding of a GRE in the promoter of target genes activates transcription, whereas binding of GR to a negative GRE (nGRE) leads to repression. Additionally, by interaction with other transcription factors, the expression pattern of GC responsive genes can be influenced in both directions*

The DNA bound receptor complex selectively recruits several coregulators (Meijer et al 2006; Rosenfeld and Glass 2001), which can either result in gene activation or gene repression. Interaction with other transcription factors, such as NF-κB or AP-1 can also result in gene repression. GR monomers interfere with these factors inhibiting their ability to activate the transcription apparatus or their ability to bind to DNA. Besides the transrepressive protein-protein interaction, synergistic effects on transactivation have been observed with, e.g., Stat-5 (Reichardt and Schütz 1998).

For regulation of the HPA axis, both DNA binding-dependent and –independent mechanisms have been observed. To separate these mechanisms, Reichhardt et al. (1998b) generated a mouse strain ($GR^{dim/dim}$) carrying a mutation in the DNA binding domain of the GR, which abolishes transactivation capability but keeps interaction with other transcription factors largely intact. In $GR^{dim/dim}$ mice, no difference was found in CRH concentration in the median eminence, indicating that regulation of CRH expression by GR is independent of DNA binding. Kretz et al. (1999) demonstrated a fivefold increase of CRH in the median eminence in mice with a targeted disruption of the GR, so that protein-protein interaction is presumably responsible for repression of CRH. POMC mRNA levels, however, were found to be strongly upregulated in the anterior pituitary of $GR^{dim/dim}$ mice and consequently levels of ACTH increased more than two-fold. These findings, together with the observation of nGREs in the POMC gene (Drouin et al 1993), further support the concept of a DNA binding-dependent mechanism in the transcriptional regulation of POMC by the GR. Another study using $GR^{dim/dim}$ mice investigated if GC actions in the hippocampus critically depend on DNA binding of GR. In $GR^{dim/dim}$ mice, in contrast to wild-type mice, corticosterone did not increase calcium current amplitudes in CA1 pyramidal neurons. Furthermore, increases in membrane hyperpolarization evoked by serotonin were not observed in $GR^{dim/dim}$ mice. Thus, the signaling pathway of GCs in the hippocampus involves DNA binding of the GR (Karst et al 2000).

Nongenomic Actions

Although evidence for rapid, non-transcriptionally mediated effects of GCs date back to the 1940s (see Dallman and Yates 1969), it was widely assumed that steroids exert their effects solely through changes in gene expression. It is now clear that GCs, like all other steroids, can rapidly alter physiological processes through nongenomic, membrane-associated mechanisms (Borski 2000). Examples of fast GC actions are found in all vertebrate classes and numerous organ systems (reviewed by Dallman 2005). In this context, effects of GCs on brain sites involved in regulation of the HPA axis will be highlighted. Fast feedback inhibition of stimulated HPA activity by GCs was first demonstrated by Dallman et al. (1969), and following *in vitro* studies supported fast feedback inhibition on CRH activity and ACTH secretion (Widmaier and Dallman 1984; see also Makara and Haller 2001 for concise review). Di et al. (2003) demonstrated an inhibiting effect of corticosterone and Dexamethasone on hypothalamic neurons. It was shown that within three minutes of GC infusion,

glutamate-induced mini post-synaptic excitatory potentials were inhibited in CRH and other neuroendocrine neurons. GCs exert these effects by stimulating endocannabinoid secretion of the target neuron, which in turn inhibits glutamatergic input to the neuron through acting on presynaptic endocannabinoid receptors. Although the downstream mechanism has been elucidated, the molecular structure of the membrane GR and the involved second messenger mechanism remain unknown (Huang et al 2006). The fact that the rapid effects of GCs can be blocked by GR antagonists (Cook 2002; Cook 2004) indicates high homology to the classical GR. It is possible that the membrane receptor represents a modified version of the classical GR capable of integrating into the membrane (Yudt and Cidlowski 2001). The membrane GR might even represent the primordial GR and the rapid effects of GCs may reflect a very ancient interaction of steroid hormones with plasma membrane receptor molecules. Taken together, the observation that GCs can rapidly elicit cell responses is consistent with the fast secretory dynamics that occur with stress and are thought to accentuate behaviors important for the adaptation of an organism under stress (Dallman 2005).

2.2.2 Glucocorticoid Receptor Structure

The human GR contains three major regions corresponding to autonomous functional domains. The N-terminal domain or transactivation domain (TAD), the central DNA-binding domain (DBD) and the C-terminal domain, also called ligand-binding domain (LBD; Encio and Detera-Wadleigh 1991; Giguere et al 1986; Weinberger et al 1985). Each domain of the GR is carrying distinct functions. Within the TAD, amino acids 77-262 encode the activating function-1 (AF-1), a fragment important for constitutive transcription and interaction with coactivators CBP and p300 (Jenkins et al 2001). The DBD plays a critical role in DNA-binding, receptor homodimerization and interaction with cofactor proteins (Freedman et al 1988; Zandi et al 1993; Zilliacus et al 1995). The LBD contains the activating function-2 (AF-2), encoded by amino acids 526 to 556, which represents the motif for ligand-dependent transactivational activity. The LBD also contains the steroid-binding region and subdomains that interact with other transcription factors (Tang et al 1998).

2.2.3 Genetic Organization

The human GR gene is located on chromosome 5 (locus 5q31-32) and is comprised of over 140 kb of nucleotides (Lu and Cidlowski 2004). It contains 8 coding exons (exons 2 to 9) and multiple alternative 5'-non-coding exon 1s (Breslin et al 2001;

Breslin and Vedeckis 1998; Turner and Muller 2005; Zong et al 1990). Exon 2 encodes the transactivation domain, the DBD is encoded by exons 3 and 4 and a total of five exons (5-9) make up the ligand-binding domain as well as the 3´-untranslated region (UTR).

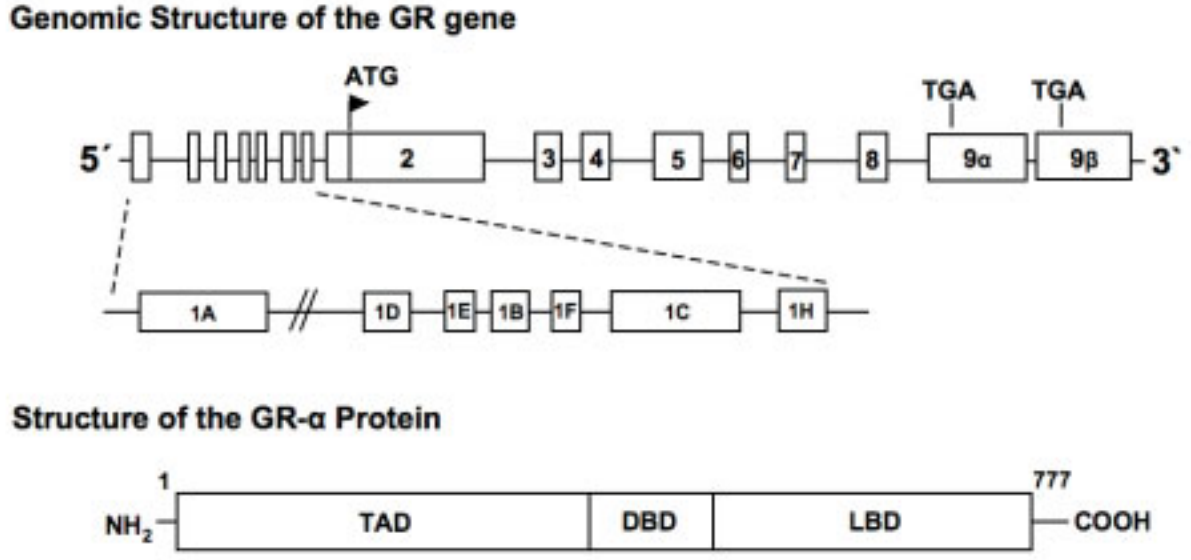

Figure 2.2: *Structure of GR gene and the full-length 777 amino acid GR-alpha protein*

Only one GR gene has been identified to date (Lu and Cidlowski 2006). Given the wide variety of responses to GCs, this observation was considered somewhat paradoxical. In part, the fact that one receptor can elicit differential downstream actions is explained by the diversity of GR isoforms. The use of different promoters, alternative splicing, alternative translation and posttranslational modifications results in considerable diversity of GR proteins, all produced from a single gene (Lu and Cidlowski 2006; Zhou and Cidlowski 2005).

2.2.4 GR Splice Variants

Multiple Exon 1s – 5´-UTR Splice Variants

Recently, analysis of the 5' end of the human GR revealed at least eleven splice variants in the 5´- (UTR), which are based on seven exon 1s (Turner and Muller 2005). All of the exon 1 variants are spliced to the same splice acceptor site in exon 2. The existence of an in-frame TGA stop codon starting 12bp before the ATG translation start site indicates that none of the exon 1 variants will be translated into amino acids (Breslin et al 2001). Thus, the predicted translated mRNA sequences from all of these variants are identical and give rise to the same protein product.

Several genes display this specific type of genomic organization, in which multiple variable first exons are each spliced to a common set of downstream constant exons to generate diverse functional mRNAs. This variable and constant genomic organization provides a genetic mechanism for directing distinct cell- and tissue-specific patterns of gene expression. This organization might provide a genetic mechanism for the diverse gene regulation required for complex mammalian development and adaptation (Zhang et al 2004).

Transcriptional Regulation

The GR is expressed in virtually all cells studied to date; however, the intracellular concentration of GR protein varies considerably among cell types. The concentration of GR is an important determinant of the type and magnitude of the cellular response to the hormone. Thus, processes that regulate the expression of the human GR gene are important in determining the cellular response to both basal and stress-induced levels of corticosteroids (Nunez and Vedeckis 2002). Although considered as housekeeping or constitutively expressed gene (Breslin and Vedeckis 1998), expression of the GR is regulated by a variety of transcription factors binding to their response elements within the promoter regions (Turner et al 2006; Yudt and Cidlowski 2002). It has been suggested that each of the untranslated first exons (see above) has a distinct proximal promoter region (Turner and Muller 2005; Turner et al 2006). By having more than one promoter sequence, GR protein expression can be differentially regulated under diverse signaling environments (Yudt and Cidlowski 2002). A number of unique binding sites for transcription factors have been identified, including those for Sp1, AP-1, YY1, NF-κB and the GR itself (Breslin et al 2001; Geng and Vedeckis 2004; Nunez and Vedeckis 2002). Although promoters of housekeeping genes generally lack a TATA box, examination of the 5´sequence revealed a TATA box upstream of exon 1-E, available for classical TATA-dependent gene regulation (Turner and Muller 2005). This variety of transcriptional regulation is thought to underlie both the cell type- and tissue-specific regulation of this widely expressed gene (Yudt and Cidlowski 2002).

GR-alpha and GR-beta Splice Variants

In addition to splice variants of the 5´-UTR, several GR splice variants that result in alternative GR protein isoforms have been observed. GR-alpha and GR-beta, as demonstrated by amino acid sequence analysis, are identical from amino acid 1 to 727 but diverge beyond this position. GR-alpha has an additional 50 amino acids and

the GR-beta form has additional nonhomologous 15 amino acids, resulting in the 35 amino acids shorter protein product. GR-alpha is the functionally active and most abundant isoform (Hagendorf et al 2005), mediating the effects of GC on transactivation and transrepression. GR-beta is generated through an alternative splicing pathway linking further downstream sequences of exon 9 to the end of exon 8. The resulting 742 AA long protein GR-beta is unable to bind ligand and is transcriptionally inactive (Yudt et al 2003). GR-beta exerts a dominant negative effect on transactivation by GR-alpha. Several mechanisms underlying this dominant negative effect have been described. Recently, Charmandari et al (2005) demonstrated that GR-beta competes with GR-alpha for binding to GRIP1 and possibly other p160 co-activators needed by GR-alpha for transactivation. In addition, competition to GRE binding (Oakley et al 1996) and the formation of transcriptionally inactive heterodimers with GR-alpha (Oakley et al 1999; Oakley et al 1996) have been suggested.

However, the biological function of GR-beta is still controversial, since very low levels of GR-beta mRNA and protein have been observed in hippocampus (DeRijk et al 2003) and other tissues (Pujols et al 2001). It has been shown that inflammatory processes (Webster et al 2001) increase the abundance of GR-beta mRNA and protein and an altered GR-alpha to GR-beta ratio has been found in GC-resistant patients (Chikanza and Kozaci 2004; Gagliardo et al 2001; Hamid et al 1999; Hori et al 2002; Zhang et al 2005). The factor necessary for alternative splicing of the GR pre-mRNA was identified as SRp30c (serine-arginine-rich protein 30c; Xu et al 2003), however, the molecular mechanisms underlying the selective generation of GR-beta by SRp30c are still unclear. Possibly, the preferential production of GR-beta depends on particular cellular contexts in which levels of this specific enzymes are upregulated, thereby directing alternative splicing pathways (Zhou and Cidlowski 2005).

GR-gamma, GR-A and GR-P

Three more GR splice variants have been detected in various tissues. The GR-gamma variant is expressed at relatively high levels of between 4-9% of the total GR transcripts in tissues like brain, lung, heart, liver, lymphocytes and muscle. The GR-gamma variant has an insertion of 3 base pairs coding for arginine between exons 3 and 4. This single amino acid insertion has been reported to decrease the transcriptional activity by nearly half (Rivers et al 1999).

The GR-A splice variant misses exons 5, 6 and 7 and has been associated with glucocorticoid-resistant phenotypes (de Lange et al 2001; Krett et al 1995; Moalli et al 1993); however, no further information is available about expression levels (Moalli et al 1993).

The GR-P variant is formed by exon 2-7 and expression of this variant has been found in various tissues and cells at levels of 10-20% of that of GR-alpha (de Lange et al 2001; Hagendorf et al 2005). The truncation of the protein results in an inability to bind GCs and GR-P exhibits lower transactivation activity than GR-alpha. Interestingly, cotransfection of the two splice variants resulted in an upregulation of GR-alpha mediated gene transcription in some, but not all cell lines (de Lange et al 2001). The mechanism of this enhancement is not understood.

2.2.5 Translational Isoforms

An additional factor contributing to tissue-selective GC responses is the formation of GR isoforms by alternative translation initiation (Lu and Cidlowski 2006). The mRNA of the GR can be translated in at least eight receptor peptides designated GR-alpha-A, -B, -C1, -C2, -C3, -D1, -D2 and D3. The GR-A isoform is the full-length 777 amino acid peptide. Translational reinitiation occurs at seven internal AUG codons in GR-alpha mRNA yielding isoforms with varying lengths of the N-terminus but identical ligand-binding domains. All observed isoforms are functional receptors and the transcriptional regulatory capabilities have been determined in luciferase reporter gene assays revealing differences in transcriptional efficacy between the isoforms. The GR-alpha-C3 isoform displays a two-fold higher transcriptional activity on GRE-driven reporter genes in various cell-lines compared with GR-A, whereas the GR-D isoforms display half of this activity (Lu and Cidlowski 2005). GR-B was reported to be nearly twice as effective in GRE mediated transactivation in a study by Yudt and Cidlowski (2001), however, the more recent report by Lu & Cidlowski (2005) did not reveal any differences between the GR-B and the full-length GR-A isoform.

Microarray analysis measuring expression of 20,186 genes in U-2 OS cells revealed that nearly 200 genes are regulated commonly by all GR-alpha isoforms. Moreover, each of the GR-alpha isoforms regulates a unique set of genes, so that the number of genes regulated by at least one of the isoforms is over 2000.

GR-alpha isoforms were found to be widely expressed in different human cell-lines and studies investigating rodents revealed that the levels of these isoforms differ widely among tissues. Lu & Cidlowski also (2005) investigated if the tissue-specific

ratios of the isoforms alter GC sensitivity. Introducing varying amounts of GR-C3 or GR-D3 into cells with constant amounts of GR-A resulted in alteration of the transcriptional potential. Thus, when GR isoforms are co-expressed, transcriptional activity reflects the composite actions of the isoforms.

2.2.6 Post-translational Modifications

Post-translational modifications of the GR protein can further modulate the transcritpional acitivity of the receptor. These modifications include phosphorylation, ubiquitination and sumoylation and play important roles in protein turnover, protein-protein interaction, the receptor's subcellular distribution and transcriptional activities (Yudt and Cidlowski 2001; Zhou and Cidlowski 2005). For example, phosphorylation enhances the transactivation activity in a gene-specific manner; therefore, different degrees of receptor phosphorylation might extend the range of the gene regulatory capability (Lu and Cidlowski 2004).

2.2.7 Polymorphisms of the GR

In the general population, a considerable variability in the response and sensitivity to GCs can be observed. Genetic variability in the form of single nucleotide polymorphisms (SNPs) and other changes at the level of the DNA sequence (insertions / deletions or repetitive elements) are thought to account for a large proportion of these differences. Genetic variations could be present in any of the genes coding for proteins or enzymes involved in GC biosynthesis (e.g. 11-, 17- and 21-hydroxylases), bioavailability (e.g. 11-beta hydroxysteroiddehydrogenases), transport (e.g. CBG), systemic absorption of GCs (e.g. P-Glycoprotein) or factors playing a role in the GR signaling pathway (e.g. heat shock proteins or co-factors). With regard to regulation of the HPA axis, even more genes involved in the direct regulation (e.g. mineralocortiocoid receptor) or indirect modulation of HPA axis activity are putative candidates. Despite the numerous factors playing a role in the exertion of GC effects, the GR itself still represents the key factor in the transduction of the GC signal and in regulation of HPA axis activity.

SNP databases such as GeneCards (http://www.genecards.org) or the SNP database of the National Center of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/SNP/) list more than 300 SNPs in the GR gene. However, most of the catalogued SNPs are either outside the coding region, do not result in an amino acid substitution or are observed at very low prevalence. A limited number of rare GR mutations result in functionally impaired protein and cause the

rare generalized inherited GC resistance syndrome (for overview, see Charmandari et al 2004). These rare mutations are not relevant for the explanation of the observed variability in GC sensitivity in the general population. The number of common polymorphisms of the GR gene that have been associated with variability in sensitivity to GCs, changes in metabolic parameters and HPA axis responses to psychosocial stress in the general population is much smaller. The relevant SNPs of the GR gene in this context (the two coding SNPs ER22/23EK and N363S, the intronic *Bcl*I and the 9beta variant in the 3´UTR) will be described below. Haplotype structure is indicated in Figure 2.3.

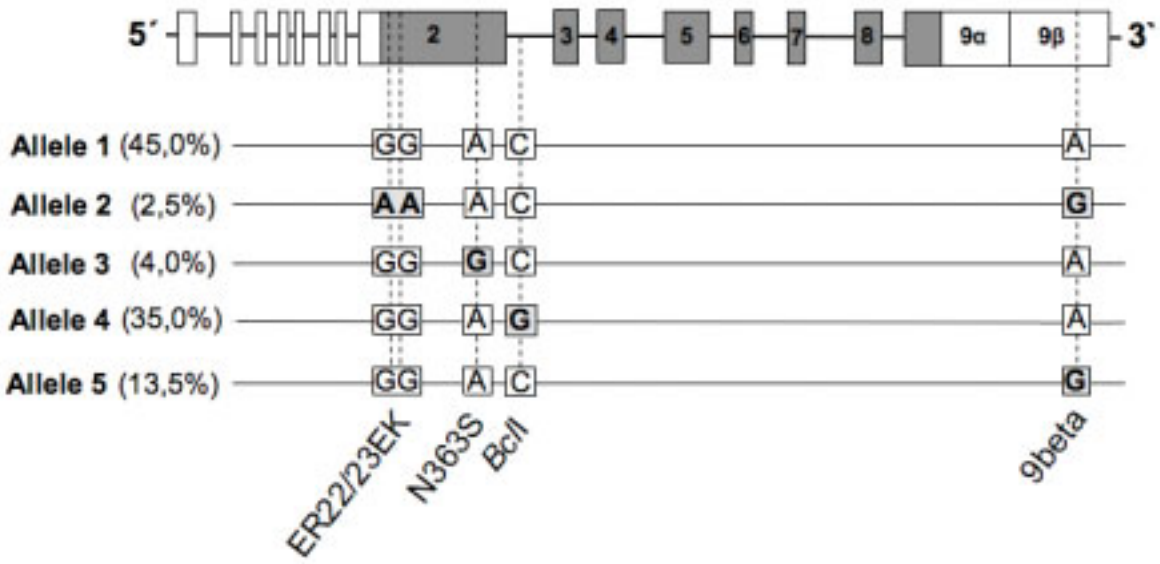

Figure 2.3: *Haplotype structure of the investigated polymorphisms. Allele 1 is the most commonly observed combination. Three of the four polymorphisms were found to be mutually exclusive (alleles 3, 4 and 5). Only the ER22/23EK polymorphism is always present in combination with the 9beta SNP (allele 2)*

***Bcl*I (no rs number assigned)**

The most extensively studied polymorphism of the GR is a *Bcl*I restriction fragment length polymorphism, identified as a C to G nucleotide change in intron B, 646 bp downstream of the 3´end of exon 2 (Fleury et al 2003; Van Rossum et al 2003). This variant was shown to be associated with measures of body composition, metabolic parameters and indices of GC sensitivity (Van Rossum and Lamberts 2004). Rosmond et al. (2000) report associations with body mass index (BMI), waist to hip ratio (WHR) and leptin and cortisol levels following a standard meal. Furthermore, the *Bcl*I was associated with indices of insulin resistance (Weaver et al 1992), abdominal

visceral fat (Buemann et al 1997) and body weight, LDL cholesterole and systolic blood pressure (Ukkola et al 2001). Decreased cortisol levels following Dexamethasone suppression (Stevens et al 2004; Van Rossum et al 2003) in *Bcl*I G carriers and reduced skin vasoconstriction after application of budenoside in *Bcl*I G homozygotes (Panarelli et al 1998) indicate tissue specific effects of this variant on GC sensitivity. Decreased HPA axis responses to a psychosocial stressor were observed in homozygous carriers of the G allele (Wüst et al 2004) and, in addition, the *Bcl*I GG genotype was associated with an increased susceptibility to develop major depression (van Rossum et al 2006). The molecular mechanism of the intronic *Bcl*I variant has not been elucidated. Possibly, the *Bcl*I is linked to another SNP in the regulatory region or in the 3´-UTR, which could result in altered expression or altered stability of the mRNA, respectively.

N363S (rs6195)

The N363S variant located in exon 2 is a coding SNP leading to a non-synonymous amino acid change from asparagine (N) to serine (S) in codon 363. In some studies, the less frequent 363S allele was associated with a higher WHR (Dobson et al 2001) and higher BMI (Di Blasio et al 2003; Lin et al 2003a) while others could not confirm this finding (Echwald et al 2001; Rosmond et al 2001). A recent meta-analysis concluded that there is no convincing evidence for an association of the N363S variant and BMI or obesity risk (Marti et al 2006). A higher incidence of coronary artery disease independent of weight, and several atherosclerosis risk factors were also associated with the N363S variant (Lin et al 2003b). *In vivo*, this polymorphism appeared to be associated with increased sensitivity to GCs, indicated by enhanced cortisol suppression and increased insulin secretion following Dexamethasone administration (Huizenga et al 1998; Koper et al 1997). Recently, this observation was further supported *in vitro* by functional studies showing the 363S allele to be more efficient in transactivating activity than the 363N allele (Russcher et al 2005a). In contrast to the *Bcl*I GG genotype, 363S carriers displayed enhanced cortisol secretion to psychosocial stress (Wüst et al 2004). The exact molecular mechanism through which the N363S SNP exerts its effects remains unknown. The amino acid change to serine might contribute a new phosphorylation site, which might alter protein interactions with transcription cofactors.

ER22/23EK (rs6189&rs6190)

The ER22/23EK polymorphism, also located in exon 2, consists of two linked SNPs in codon 22 and 23 separated by 1bp (Koper et al 1997). The base change in codon

22 is silent with both GAG and GAA coding for glutamic acid, whereas the change from AGG to AAG in codon 23 leads to an amino acid change from arginine (R) to lysine (K). While the N363S variant seems to increase sensitivity to GCs, the ER22/23EK polymorphism was found to reduce sensitivity to GCs. Functional analyses revealed that the ER22/23EK polymorphism leads to a higher expression of the translational GR-A isoform at the expense of the GR-B, which has higher transactivating capacity (Russcher et al 2005b). This shift in GR-A to GR-B expression ratio was thought to result in a reduced transactivating capacity of the 22/23EK allele, which has been confirmed in yet another study (Russcher et al 2005a). Overall, findings suggest that carriers of the ER22/23EK polymorphism have a healthier cardiovascular and metabolic profile, indicated by lower total and low-density lipoprotein cholesterol levels, lower fasting insulin concentration, better insulin sensitivity and lower C-reactive protein levels (Van Rossum et al 2004; van Rossum et al 2002). Furthermore, fewer white matter lesions in the brain and a lower risk of dementia have been observed in 22/23EK carriers and the prevalence of this polymorphism was higher in an older population, possibly indicating an effect on survival (Van Rossum et al 2004; Van Rossum and Lamberts 2004). Two recent studies (van Rossum et al 2006; van West et al 2006) independently reported an association of the ER22/23EK polymorphism with major depression.

9beta (rs6198)

Another common variant of the GR is an A to G transition at position 3669 in the 3´untranslated region (UTR) at the end of exon 9beta. Functional studies revealed a stabilizing effect of this polymorphism on GR-beta mRNA *in vitro*, possibly leading to enhanced expression of GR-beta protein (Derijk et al 2001). GR-beta protein is unable to bind ligand and acts as a dominant negative inhibitor of GR-alpha (Charmandari et al 2005; Oakley et al 1999; Schaaf and Cidlowski 2002; Yudt et al 2003). Enhanced expression of the GR-beta was associated with GC resistance in allergic disease (Hamid et al 1999; Leung et al 1997; Sousa et al 2000) and a study with rheumatoid arthritis patients showed a higher prevalence of the 9beta variant in patients than in controls (Derijk et al 2001). Furthermore, a lower transrepressing activity has been reported for this variant (van den Akker et al 2006b) and a recent study observed a lower risk of persistent *Staphylococcus Aureus* nasal carriage in 9beta GG carriers, possibly resulting from reduced GC-induced immune suppression (van den Akker et al 2006a). Further support for a relative GC resistance in 9beta G allele carriers stems from the observation of reduced central obesity in Europid

women and a more favorable lipid profile in Europid men carrying this polymorphism (Syed et al 2006).

2.3 References

Abelson JL, Curtis GC (1996): Hypothalamic-pituitary-adrenal axis activity in panic disorder. 24-hour secretion of corticotropin and cortisol. *Arch Gen Psychiatry* 53:323-331.

Aranda A, Pascual A (2001): Nuclear hormone receptors and gene expression. *Physiol Rev* 81:1269-1304.

Borski RJ (2000): Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends Endocrinol Metab* 11:427-436.

Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ (2005): Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proc Natl Acad Sci U S A* 102:473-478.

Breslin MB, Geng CD, Vedeckis WV (2001): Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. *Mol Endocrinol* 15:1381-1395.

Breslin MB, Vedeckis WV (1998): The human glucocorticoid receptor promoter upstream sequences contain binding sites for the ubiquitous transcription factor, Yin Yang 1. *J Steroid Biochem Mol Biol* 67:369-381.

Bridgham JT, Carroll SM, Thornton JW (2006): Evolution of hormone-receptor complexity by molecular exploitation. *Science* 312:97-101.

Buemann B, Vohl MC, Chagnon M, Chagnon YC, Gagnon J, Perusse L, et al (1997): Abdominal visceral fat is associated with a *Bcl*I restriction fragment length polymorphism at the glucocorticoid receptor gene locus. *Obes Res* 5:186-192.

Cannon WB (1914): The interrelations of emotions as suggested by recent physiological researches. *Am J Psychology* 25:256.

Cannon WB (1915): *Bodily changes in pain, hunger, fear and rage*. New York, London: Applegate and Co.

Cannon WB (1932): *Wisdom of the body*. New York: Norton.

Charmandari E, Chrousos GP, Ichijo T, Bhattacharyya N, Vottero A, Souvatzoglou E, Kino T (2005): The human glucocorticoid receptor (hGR) beta isoform suppresses the transcriptional activity of hGRalpha by interfering with formation of active coactivator complexes. *Mol Endocrinol* 19:52-64.

Charmandari E, Kino T, Chrousos GP (2004): Familial/sporadic glucocorticoid resistance: clinical phenotype and molecular mechanisms. *Ann N Y Acad Sci* 1024:168-181.

Chikanza IC, Kozaci DL (2004): Corticosteroid resistance in rheumatoid arthritis: molecular and cellular perspectives. *Rheumatology (Oxford)* 43:1337-1345.

Chrousos GP, Gold PW (1992): The concepts of stress and stress system disorders. *Journal of the American Medical Association* 267:1244-1252.

Cole MA, Kalman BA, Pace TW, Topczewski F, Lowrey MJ, Spencer RL (2000): Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. *J Neuroendocrinol* 12:1034-1042.

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, et al (1995): Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev* 9:1608-1621.

Cole TJ, Harris HJ, Hoong I, Solomon N, Smith R, Krozowski Z, Fullerton MJ (1999): The glucocorticoid receptor is essential for maintaining basal and dexamethasone-induced repression of the murine corticosteroid-binding globulin gene. *Mol Cell Endocrinol* 154:29-36.

Cole TJ, Myles K, Purton JF, Brereton PS, Solomon NM, Godfrey DI, Funder JW (2001): GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant. *Mol Cell Endocrinol* 173:193-202.

Cook CJ (2002): Glucocorticoid feedback increases the sensitivity of the limbic system to stress. *Physiol Behav* 75:455-464.

Cook CJ (2004): Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala. *Physiol Behav* 82:751-762.

Daban C, Vieta E, Mackin P, Young AH (2005): Hypothalamic-pituitary-adrenal axis and bipolar disorder. *Psychiatr Clin North Am* 28:469-480.

Dallman MF (2005): Fast glucocorticoid actions on brain: back to the future. *Front Neuroendocrinol* 26:103-108.

Dallman MF, Yates FE (1969): Dynamic asymmetries in the corticosteroid feedback path and distribution-metabolism-binding elements of the adrenocortical system. *Ann N Y Acad Sci* 156:696-721.

Datson NA, van der Perk J, de Kloet ER, Vreugdenhil E (2001): Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. *Eur J Neurosci* 14:675-689.

de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475.

De Kloet ER, Reul JM (1987): Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. *Psychoneuroendocrinology* 12:83-105.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998): Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 19:269-301.

de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO, et al (2001): Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. *Cancer Res* 61:3937-3941.

Degen SB, Verheij MM, Cools AR (2004): Genetic background, nature of event, and time of exposure to event direct the phenotypic expression of a particular genotype. A study with apomorphine-(un)susceptible Wistar rats. *Behav Brain Res* 154:107-112.

DeRijk RH, Schaaf M, Stam FJ, de Jong IE, Swaab DF, Ravid R, et al (2003): Very low levels of the glucocorticoid receptor beta isoform in the human hippocampus as shown by Taqman RT-PCR and immunocytochemistry. *Brain Res Mol Brain Res* 116:17-26.

Derijk RH, Schaaf MJ, Turner G, Datson NA, Vreugdenhil E, Cidlowski J, et al (2001): A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol* 28:2383-2388.

Di Blasio AM, van Rossum EF, Maestrini S, Berselli ME, Tagliaferri M, Podesta F, et al (2003): The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients. *Clin Endocrinol (Oxf)* 59:68-74.

Di S, Malcher-Lopes R, Halmos KC, Tasker JG (2003): Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. *J Neurosci* 23:4850-4857.

Dobson MG, Redfern CP, Unwin N, Weaver JU (2001): The N363S polymorphism of the glucocorticoid receptor: potential contribution to central obesity in men and lack of association with other risk factors for coronary heart disease and diabetes mellitus. *J Clin Endocrinol Metab* 86:2270-2274.

Drouin J, Sun YL, Chamberland M, Gauthier Y, De Lean A, Nemer M, Schmidt TJ (1993): Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. *Embo J* 12:145-156.

Ebrecht M, Buske-Kirschbaum A, Hellhammer D, Kern S, Rohleder N, Walker B, Kirschbaum C (2000): Tissue specificity of glucocorticoid sensitivity in healthy adults. *J Clin Endocrinol Metab* 85:3733-3739.

Echwald SM, Sorensen TI, Andersen T, Pedersen O (2001): The Asn363Ser variant of the glucocorticoid receptor gene is not associated with obesity or weight gain in Danish men. *Int J Obes Relat Metab Disord* 25:1563-1565.

Encio IJ, Detera-Wadleigh SD (1991): The genomic structure of the human glucocorticoid receptor. *J Biol Chem* 266:7182-7188.

Entringer S, Wüst S, Kumsta R, Hellhamer DH, Wadhwa PD (in prep): Exposure to maternal psychosocial stress in utero and HPA axis function in adult life.

Feldker DE, Datson NA, Veenema AH, Meulmeester E, de Kloet ER, Vreugdenhil E (2003): Serial analysis of gene expression predicts structural differences in hippocampus of long attack latency and short attack latency mice. *Eur J Neurosci* 17:379-387.

Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, et al (1999): Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. *Development* 126:2935-2944.

Fleury I, Beaulieu P, Primeau M, Labuda D, Sinnett D, Krajinovic M (2003): Characterization of the *Bcl*I polymorphism in the glucocorticoid receptor gene. *Clin Chem* 49:1528-1531.

Francis D, Diorio J, Liu D, Meaney MJ (1999): Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* 286:1155-1158.

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR (1988): The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* 334:543-546.

Gagliardo R, Vignola AM, Mathieu M (2001): Is there a role for glucocorticoid receptor beta in asthma? *Respir Res* 2:1-4.

Geng CD, Vedeckis WV (2004): Steroid-responsive sequences in the human glucocorticoid receptor gene 1A promoter. *Mol Endocrinol* 18:912-924.

Gesing A, Bilang-Bleuel A, Droste SK, Linthorst AC, Holsboer F, Reul JM (2001): Psychological stress increases hippocampal mineralocorticoid receptor levels: involvement of corticotropin-releasing hormone. *J Neurosci* 21:4822-4829.

Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM (1986): Functional domains of the human glucocorticoid receptor. *Cell* 46:645-652.

Hagendorf A, Koper JW, de Jong FH, Brinkmann AO, Lamberts SW, Feelders RA (2005): Expression of the human glucocorticoid receptor splice variants alpha, beta, and P in peripheral blood mononuclear leukocytes in healthy controls and in patients with hyper- and hypocortisolism. *J Clin Endocrinol Metab* 90:6237-6243.

Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ, et al (1999): Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 159:1600-1604.

Heim C, Ehlert U, Hellhammer DH (2000): The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology* 25:1-35.

Heim C, Newport DJ, Wagner D, Wilcox MM, Miller AH, Nemeroff CB (2002): The role of early adverse experience and adulthood stress in the prediction of neuroendocrine stress reactivity in women: a multiple regression analysis. *Depress Anxiety* 15:117-125.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003): Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol* 24:151-180.

Holsboer F (2000): The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* 23:477-501.

Hori T, Watanabe K, Miyaoka M, Moriyasu F, Onda K, Hirano T, Oka K (2002): Expression of mRNA for glucocorticoid receptors in peripheral blood mononuclear cells of patients with Crohn's disease. *J Gastroenterol Hepatol* 17:1070-1077.

Howell MP, Muglia LJ (2006): Effects of genetically altered brain glucocorticoid receptor action on behavior and adrenal axis regulation in mice. *Front Neuroendocrinol*.

Huang MH, So EC, Liu YC, Wu SN (2006): Glucocorticoids stimulate the activity of large-conductance Ca2+ -activated K+ channels in pituitary GH3 and AtT-20 cells via a non-genomic mechanism. *Steroids* 71:129-140.

Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, et al (1998): A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *J Clin Endocrinol Metab* 83:144-151.

Jacobson L, Sapolsky R (1991): The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews* 12:118-134.

Jenkins BD, Pullen CB, Darimont BD (2001): Novel glucocorticoid receptor coactivator effector mechanisms. *Trends Endocrinol Metab* 12:122-126.

Karst H, Berger S, Turiault M, Tronche F, Schutz G, Joels M (2005): Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proc Natl Acad Sci U S A* 102:19204-19207.

Karst H, Karten YJ, Reichardt HM, de Kloet ER, Schutz G, Joels M (2000): Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. *Nat Neurosci* 3:977-978.

Kendler KS, Bulik CM, Silberg J, Hettema JM, Myers J, Prescott CA (2000): Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. *Arch Gen Psychiatry* 57:953-959.

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, et al (1997): Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. *Hum Genet* 99:663-668.

Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005): The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci Biobehav Rev* 29:3-38.

Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST (1995): A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. *Cancer Res* 55:2727-2729.

Kretz O, Reichardt HM, Schutz G, Bock R (1999): Corticotropin-releasing hormone expression is the major target for glucocorticoid feedback-control at the hypothalamic level. *Brain Res* 818:488-491.

Landgraf R, Wigger A (2003): Born to be anxious: neuroendocrine and genetic correlates of trait anxiety in HAB rats. *Stress* 6:111-119.

Laudet V (1997): Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* 19:207-226.

Lazarus RS, Folkman, S. (1984): *Stress, appraisal, and coping*. New York: Springer.

Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, et al (1997): Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* 186:1567-1574.

Levine S (1957): Infantile experience and resistance to physiological stress. *Science* 126:405.

Levine S (2003): Stress: An historical perspective. In: Steckler T, Kalin, N. & Reul, J.M.H.M. editor. *Handbook on Stress, Immunology and Behavior*. Amsterdam: Elsevier, pp 3-23.

Levine S, Ursin, H. (1991): What is stress? In: Bown MR, Koob, G.F. editor. *Stress, Neurobiology and Neuroendocrinology*. New York: Marcel Dekker, Inc., pp 3-21.

Lin RC, Wang XL, Dalziel B, Caterson ID, Morris BJ (2003a): Association of obesity, but not diabetes or hypertension, with glucocorticoid receptor N363S variant. *Obes Res* 11:802-808.

Lin RC, Wang XL, Morris BJ (2003b): Association of coronary artery disease with glucocorticoid receptor N363S variant. *Hypertension* 41:404-407.

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, et al (1997): Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 277:1659-1662.

Lu NZ, Cidlowski JA (2004): The origin and functions of multiple human glucocorticoid receptor isoforms. *Ann N Y Acad Sci* 1024:102-123.

Lu NZ, Cidlowski JA (2005): Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell* 18:331-342.

Lu NZ, Cidlowski JA (2006): Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol* 16:301-307.

Makara GB, Haller J (2001): Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. *Prog Neurobiol* 65:367-390.

Marti A, Ochoa MC, Sanchez-Villegas A, Martinez JA, Martinez-Gonzalez MA, Hebebrand J, et al (2006): Meta-analysis on the effect of the N363S polymorphism of the glucocorticoid receptor gene (GRL) on human obesity. *BMC Med Genet* 7:50.

Mason JW (1968): A review of psychoendocrine research on the pituitary-adrenal cortical system. *Psychosomatic Medicine* 30:576-607.

McEwen BS (1998): Stress, adaptation, and disease. Allostasis and allostatic load. *Ann N Y Acad Sci* 840:33-44.

McEwen BS (2000): Stress, Definition and concepts of. In: Fink G editor. *Encyclopedia of Stress*, Vol 3. San Diego: Academic Press, pp 508-509.

Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM (1988): Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 239:766-768.

Meijer OC, van der Laan S, Lachize S, Steenbergen PJ, de Kloet ER (2006): Steroid receptor coregulator diversity: what can it mean for the stressed brain? *Neuroscience* 138:891-899.

Moalli PA, Pillay S, Krett NL, Rosen ST (1993): Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells. *Cancer Res* 53:3877-3879.

Morsink MC, Steenbergen PJ, Vos JB, Karst H, Joels M, De Kloet ER, Datson NA (2006): Acute activation of hippocampal glucocorticoid receptors results in different waves of gene expression throughout time. *J Neuroendocrinol* 18:239-252.

Nunez BS, Vedeckis WV (2002): Characterization of promoter 1B in the human glucocorticoid receptor gene. *Mol Cell Endocrinol* 189:191-199.

Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA (1999): The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem* 274:27857-27866.

Oakley RH, Sar M, Cidlowski JA (1996): The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *J Biol Chem* 271:9550-9559.

Oitzl MS, Reichardt HM, Joels M, de Kloet ER (2001): Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. *Proc Natl Acad Sci U S A* 98:12790-12795.

Panarelli M, Holloway CD, Fraser R, Connell JM, Ingram MC, Anderson NH, Kenyon CJ (1998): Glucocorticoid receptor polymorphism, skin vasoconstriction, and other metabolic intermediate phenotypes in normal human subjects. *J Clin Endocrinol Metab* 83:1846-1852.

Pariante CM (2003): Depression, stress and the adrenal axis. *J Neuroendocrinol* 15:811-812.

Pariante CM, Miller AH (2001): Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* 49:391-404.

Pariante CM, Nemeroff CB, Miller AH (1995): Glucocorticoid receptors in depression. *Isr J Med Sci* 31:705-712.

Perlman WR, Webster MJ, Kleinman JE, Weickert CS (2004): Reduced glucocorticoid and estrogen receptor alpha messenger ribonucleic acid levels in the amygdala of patients with major mental illness. *Biol Psychiatry* 56:844-852.

Pujols L, Mullol J, Perez M, Roca-Ferrer J, Juan M, Xaubet A, et al (2001): Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. *Am J Respir Cell Mol Biol* 24:49-57.

Raison CL, Miller AH (2003): When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. *Am J Psychiatry* 160:1554-1565.

Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al (1998a): DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 93:531-541.

Reichardt HM, Kaestner KH, Wessely O, Gass P, Schmid W, Schutz G (1998b): Analysis of glucocorticoid signalling by gene targeting. *J Steroid Biochem Mol Biol* 65:111-115.

Reichardt HM, Schutz G (1998): Glucocorticoid signalling--multiple variations of a common theme. *Mol Cell Endocrinol* 146:1-6.

Reul JM, Gesing A, Droste S, Stec IS, Weber A, Bachmann C, et al (2000): The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. *Eur J Pharmacol* 405:235-249.

Reul JMHM, De Kloet ER (1985): Two receptor systems for corticosterone in rat brain: microdistribution and differential occpuation. *Endocrinology* 117:2505-2511.

Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, et al (2005): Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J Neurosci* 25:6243-6250.

Rivers C, Levy A, Hancock J, Lightman S, Norman M (1999): Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing. *J Clin Endocrinol Metab* 84:4283-4286.

Rosenfeld MG, Glass CK (2001): Coregulator codes of transcriptional regulation by nuclear receptors. *J Biol Chem* 276:36865-36868.

Rosmond R, Bouchard C, Bjorntorp P (2001): Tsp509I polymorphism in exon 2 of the glucocorticoid receptor gene in relation to obesity and cortisol secretion: cohort study. *Bmj* 322:652-653.

Rosmond R, Chagnon YC, Holm G, Chagnon M, Perusse L, Lindell K, et al (2000): A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. *Obes Res* 8:211-218.

Russcher H, Smit P, van den Akker EL, van Rossum EF, Brinkmann AO, de Jong FH, et al (2005a): Two Polymorphisms in the Glucocorticoid Receptor Gene Directly Affect Glucocorticoid-Regulated Gene Expression. *J Clin Endocrinol Metab*.

Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW (2005b): Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. *Mol Endocrinol* 19:1687-1696.

Ryan MC, Sharifi N, Condren R, Thakore JH (2004): Evidence of basal pituitary-adrenal overactivity in first episode, drug naive patients with schizophrenia. *Psychoneuroendocrinology* 29:1065-1070.

Rybakowski JK, Twardowska K (1999): The dexamethasone/corticotropin-releasing hormone test in depression in bipolar and unipolar affective illness. *J Psychiatr Res* 33:363-370.

Sapolsky R (1994): *Why Zebras Don´t Get Ulcers*. New York: W.H. Freeman and Co.

Sapolsky RM, Romero LM, Munck AU (2000): How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21:55-89.

Schaaf MJ, Cidlowski JA (2002): AUUUA motifs in the 3'UTR of human glucocorticoid receptor alpha and beta mRNA destabilize mRNA and decrease receptor protein expression. *Steroids* 67:627-636.

Seckl JR, Meaney MJ (2004): Glucocorticoid programming. *Ann N Y Acad Sci* 1032:63-84.

Selye H (1936): A syndrome produced by diverse nocuous agents. *Nature* 38:32.

Selye H (1956): *The Stress of life*. New York: McGraw-Hill.

Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ, Lee TH (2000): Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol* 105:943-950.

Steptoe A (2000): Stress effects, overview. In: Fink G editor. *Encyclopedia of Stress*, Vol 3. San Diego: Academic Press, pp 510-511.

Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE, Donn R (2004): Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. *J Clin Endocrinol Metab* 89:892-897.

Syed AA, Irving JA, Redfern CP, Hall AG, Unwin NC, White M, et al (2006): Association of glucocorticoid receptor polymorphism A3669G in exon 9beta with reduced central adiposity in women. *Obesity (Silver Spring)* 14:759-764.

Tang Y, Getzenberg RH, Vietmeier BN, Stallcup MR, Eggert M, Renkawitz R, DeFranco DB (1998): The DNA-binding and tau2 transactivation domains of the rat glucocorticoid receptor constitute a nuclear matrix-targeting signal. *Mol Endocrinol* 12:1420-1431.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, et al (1999): Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99-103.

Turner JD, Muller CP (2005): Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. *J Mol Endocrinol* 35:283-292.

Turner JD, Schote AB, Macedo JA, Pelascini LP, Muller CP (2006): Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage? *Biochem Pharmacol*.

Ukkola O, Rosmond R, Tremblay A, Bouchard C (2001): Glucocorticoid receptor *Bcl* I variant is associated with an increased atherogenic profile in response to long-term overfeeding. *Atherosclerosis* 157:221-224.

van den Akker EL, Nouwen JL, Melles DC, Rossum EF, Koper JW, Uitterlinden AG, et al (2006a): Staphylococcus aureus Nasal Carriage Is Associated with Glucocorticoid Receptor Gene Polymorphisms. *J Infect Dis* 194:814-818.

van den Akker EL, Russcher H, van Rossum EF, Brinkmann AO, de Jong FH, Hokken A, et al (2006b): Glucocorticoid receptor polymorphism affects transrepression but not transactivation. *J Clin Endocrinol Metab* 91:2800-2803.

van Rossum EF, Binder EB, Majer M, Koper JW, Ising M, Modell S, et al (2006): Polymorphisms of the glucocorticoid receptor gene and major depression. *Biol Psychiatry* 59:681-688.

Van Rossum EF, Feelders RA, Van Den Beld AW, Uitterlinden AG, Janssen JA, Ester W, et al (2004): Association of the ER22/23EK polymorphism in the glucocorticoid receptor gene with survival and C-reactive protein levels in elderly men. *Am J Med* 117:158-162.

van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, et al (2002): A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 51:3128-3134.

Van Rossum EF, Koper JW, Van Den Beld AW, Uitterlinden AG, Arp P, Ester W, et al (2003): Identification of the *Bcl*I polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. *Clin Endocrinol (Oxf)* 59:585-592.

Van Rossum EF, Lamberts SW (2004): Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. *Recent Prog Horm Res* 59:333-357.

van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, et al (2006): Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. *Neuropsychopharmacology* 31:620-627.

Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM (2003): Genetic selection for coping style predicts stressor susceptibility. *J Neuroendocrinol* 15:256-267.

Weaver JU, Hitman GA, Kopelman PG (1992): An association between a BcI1 restriction fragment length polymorphism of the glucocorticoid receptor locus and hyperinsulinaemia in obese women. *J Mol Endocrinol* 9:295-300.

Webster JC, Oakley RH, Jewell CM, Cidlowski JA (2001): Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. *Proc Natl Acad Sci U S A* 98:6865-6870.

Webster MJ, Knable MB, O'Grady J, Orthmann J, Weickert CS (2002): Regional specificity of brain glucocorticoid receptor mRNA alterations in subjects with schizophrenia and mood disorders. *Mol Psychiatry* 7:985-994, 924.

Wei Q, Lu XY, Liu L, Schafer G, Shieh KR, Burke S, et al (2004): Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. *Proc Natl Acad Sci U S A* 101:11851-11856.

Weinberger C, Hollenberg SM, Ong ES, Harmon JM, Brower ST, Cidlowski J, et al (1985): Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. *Science* 228:740-742.

Widmaier EP, Dallman MF (1984): The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perifused pituitaries in vitro: rapid inhibition by glucocorticoids. *Endocrinology* 115:2368-2374.

Wüst S, Entringer S, Federenko IS, Schlotz W, Hellhammer DH (2005): Birth weight is associated with salivary cortisol responses to psychosocial stress in adult life. *Psychoneuroendocrinology* 30:591-598.

Wüst S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH (2004): Common Polymorphisms in the Glucocorticoid Receptor Gene Are Associated with Adrenocortical Responses to Psychosocial Stress. *J Clin Endocrinol Metab* 89:565-573.

Xu Q, Leung DY, Kisich KO (2003): Serine-arginine-rich protein p30 directs alternative splicing of glucocorticoid receptor pre-mRNA to glucocorticoid receptor beta in neutrophils. *J Biol Chem* 278:27112-27118.

Yehuda R (1997): Sensitization of the hypothalamic-pituitary-adrenal axis in posttraumatic stress disorder. *Ann N Y Acad Sci* 821:57-75.

Yehuda R (2002): Current status of cortisol findings in post-traumatic stress disorder. *Psychiatr Clin North Am* 25:341-368, vii.

Yehuda R, Golier JA, Yang RK, Tischler L (2004): Enhanced sensitivity to glucocorticoids in peripheral mononuclear leukocytes in posttraumatic stress disorder. *Biol Psychiatry* 55:1110-1116.

Yehuda R, Halligan SL, Grossman R, Golier JA, Wong C (2002): The cortisol and glucocorticoid receptor response to low dose dexamethasone administration in aging combat veterans and holocaust survivors with and without posttraumatic stress disorder. *Biol Psychiatry* 52:393-403.

Young AH (2004): Cortisol in mood disorders. *Stress* 7:205-208.

Yudt MR, Cidlowski JA (2001): Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Mol Endocrinol* 15:1093-1103.

Yudt MR, Cidlowski JA (2002): The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. *Mol Endocrinol* 16:1719-1726.

Yudt MR, Jewell CM, Bienstock RJ, Cidlowski JA (2003): Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* 23:4319-4330.

Zandi E, Galli I, Dobbeling U, Rusconi S (1993): Zinc finger mutations that alter domain interactions in the glucocorticoid receptor. *J Mol Biol* 230:124-136.

Zhang H, Ouyang Q, Wen ZH, Fiocchi C, Liu WP, Chen DY, Li FY (2005): Significance of glucocorticoid receptor expression in colonic mucosal cells of patients with ulcerative colitis. *World J Gastroenterol* 11:1775-1778.

Zhang T, Haws P, Wu Q (2004): Multiple variable first exons: a mechanism for cell- and tissue-specific gene regulation. *Genome Res* 14:79-89.

Zhou J, Cidlowski JA (2005): The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids* 70:407-417.

Zilliacus J, Wright AP, Carlstedt-Duke J, Gustafsson JA (1995): Structural determinants of DNA-binding specificity by steroid receptors. *Mol Endocrinol* 9:389-400.

Zong J, Ashraf J, Thompson EB (1990): The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. *Mol Cell Biol* 10:5580-5585.

CHAPTER 3

Sex Specific Associations between Common Glucocorticoid Receptor Gene Variants and Hypothalamus-Pituitary-Adrenal Axis Responses to Psychosocial Stress

3.1 Summary

Background: Alterations in glucocorticoid signaling have been associated with a number of psychiatric disorders. Genetic variation of the glucocorticoid receptor (GR) might be one of the factors underlying susceptibility to stress related disease.

Methods: We investigated 206 healthy subjects and assessed the association between four common GR gene polymorphisms (ER22/23EK, N363S, *Bcl*I, 9beta) and HPA axis responses to psychosocial stress (Trier Social Stress Test, TSST).

Results: Male 9beta AG carriers displayed the highest ACTH and total cortisol levels after TSST exposure whereas male *Bcl*I GG carriers showed diminished responses (for ACTH: main effect *genotype* p=.05). Remarkably, the *Bcl*I GG genotype in women was associated with the highest total cortisol TSST responses, resulting in a significant *sex* by *genotype* interaction (p=.03).

Conclusions: We observed significant sex specific associations between GR gene polymorphisms and HPA axis responses to psychosocial stress. These findings support the relevance of GR gene polymorphisms in HPA axis regulation. Genetic variations of the GR might constitute a risk factor in development of HPA related psychiatric disorders.

3.2 Introduction

In response to stress, a wide spectrum of adaptive autonomic, physiological and behavioral responses are triggered, including activation of the hypothalamus-pituitary-adrenal (HPA) axis. Cortisol, secreted by the adrenals upon activation of the HPA axis, has diverse regulatory roles in development, metabolism and behavior as well as in control of the HPA axis itself. The actions of cortisol are primarily mediated by the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily. The GR exerts its effects by transactivation or repression of glucocorticoid (GC) responsive genes by direct DNA binding or by interaction with other transcription factors (Bamberger et al 1996; Reichardt and Schutz 1998). One important function of the GR is the exertion of negative feedback on HPA axis activity and hence termination of the stress response (de Kloet 2005; Jacobson and Sapolsky 1991). Impaired functioning of HPA axis regulation has been associated with several psychosomatic and psychiatric disorders, such as depression, the metabolic syndrome, fibromyalgia and post traumatic stress disorder (Bjorntorp and Rosmond 2000; Holsboer 2001; Parker et al 2001; Yehuda 1997). Genetic variation of the GR might be one of the factors underlying individual susceptibility to stress related disorders. In a growing number of studies, common polymorphisms of the GR gene have been associated with variability in sensitivity to GCs, changes in metabolic parameters and HPA axis responses to psychosocial stress. The common polymorphism *Bcl*I (no rs number available) located in intron B (Fleury et al 2003; Van Rossum et al 2003) has been extensively studied and was shown to be associated with measures of body composition, metabolic parameters and indices of GC sensitivity (see Van Rossum and Lamberts 2004 for review). Decreased HPA axis responses to a psychosocial stressor were observed in homozygous carriers of the G allele (Wüst et al 2004) and, in addition, the *Bcl*I GG genotype was associated with an increased susceptibility to develop major depression (van Rossum et al 2006).

The N363S variant (rs6195), located in exon 2, is a coding single nucleotide polymorphism (SNP) leading to a non-synonymous amino acid change from asparagine (N) to serine (S) in codon 363. In some studies, the rarer 363S allele was associated with a higher waist to hip ratio (Dobson et al 2001) and higher body mass index (Di Blasio et al 2003; Lin et al 1999; Lin et al 2003) while others could not

confirm this finding (Echwald et al 2001; Rosmond et al 2000). This polymorphism appeared to be associated with increased sensitivity to GCs as indicated by enhanced cortisol suppression and increased insulin secretion following Dexamethasone administration (Huizenga et al 1998; Koper et al 1997). Functional *in vitro* studies showed that the 363S allele is more efficient in transactivating activity, but not transrepression, than the 363N allele (Russcher et al 2005a). In contrast to the *Bcl*I GG genotype, 363S carriers displayed enhanced cortisol secretion to psychosocial stress (Wüst et al 2004).

The ER22/23EK polymorphism (rs6189 & rs6190), also located in exon 2, consists of two linked SNPs in codon 22 and 23 separated by one base pair (Koper et al 1997). The base change in codon 22 is silent, whereas the change from AGG to AAG in codon 23 leads to an amino acid change from arginine (R) to lysine (K). The 22/23EK allele was found to reduce sensitivity to the exogenous GC Dexamethasone. *In vitro* analyses revealed that the ER22/23EK polymorphism leads to a higher expression of the translational GR-A isoform at the expense of the GR-B, which results in a reduced transactivating capacity of the 22/23EK allele (Russcher et al 2005b). Overall, findings suggest that carriers of the ER22/23EK polymorphism have a healthier cardiovascular and metabolic profile, fewer white matter lesions in the brain and a lower risk of dementia (for review van Rossum and Lamberts 2004). Furthermore, the prevalence of this polymorphism was higher in an older population, possibly indicating an effect on survival (Van Rossum et al 2004a). Two recent studies (van Rossum et al 2006; van West et al 2006) independently reported an association of the ER22/23EK polymorphism with major depression.

Another common variant of the GR is an A to G transition in the 3´untranslated region (UTR) at the end of exon 9beta (rs6198). Functional studies revealed a stabilizing effect of this polymorphism on GRbeta mRNA *in vitro*, possibly leading to enhanced expression of GRbeta protein (Derijk et al 2001). GRbeta protein is unable to bind ligand and acts as a dominant negative inhibitor of GRalpha (Charmandari et al 2005; Yudt et al 2003). Enhanced expression of the GRbeta was associated with GC resistance in allergic disease (Bamberger et al 1995; Hamid et al 1999; Leung et al 1997; Sousa et al 2000; Webster et al 2001) and a study with rheumatoid arthritis patients showed a higher prevalence of the 9beta G variant in patients than in controls (Derijk et al 2001).

Given this evidence, functional relevance of these gene variants for GC sensitivity seems obvious. However, knowledge on the influence of these variants on HPA axis

response to challenge is limited. Thus, the aim of the current study was to estimate the spectrum of variability in HPA axis reactivity with a focus on responses to psychosocial stress that can be attributed to variants of the GR gene. All GR gene polymorphisms with known functionality or previously reported associations and sufficient prevalence in the population (ER22/23EK, N363S, *Bcl*I, 9beta) were investigated and cortisol and ACTH responses to psychosocial stress (Trier Social Stress Test) were assessed.

Since we investigated variations in just one gene with regard to the functioning of a complex system, which is modulated by numerous interacting factors, the effect sizes that can be attributed to single variants are likely to be small. However, based on previous findings, we hypothesized that comparison of GR gene variant groups with the most extreme HPA axis responses will reveal significant differences thus pointing towards a substantial relevance of GR gene polymorphisms for the variability of HPA axis regulation in a given population.

3.3 Methods and Materials

3.3.1 Subjects

Initially, 601 healthy subjects were recruited from a Trier-based community sample and from students of the University of Trier. These subjects were genotyped for the GR variants under investigation. Subsequently, 206 subjects were selected for phenotyping according to their GR genotype, which resulted in a stratified sample of about equally sized comparison groups (see Results). This sample consisted of 118 females and 88 males (mean age 25.1 with SEM of ± 3.9 yrs, BMI 24.2 ± 4.1). Participants were non-smokers and of central European descent. All females were using ethinyl-estradiol containing oral contraceptives (OC) to avoid modulation of cortisol responses to the TSST by the menstrual cycle phase (Kirschbaum et al 1999). Except for the use of OC, all subjects reported to be medication free. Prior to experimental sessions, the absence of acute or chronic diseases was confirmed in a medical exam. The protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants.

3.3.2 Experimental Protocol

Participants of the initial sample reported to our laboratory for collection of a blood sample for later genotype analysis. Subjects selected for further testing were

exposed to the Trier Social Stress Test (TSST), which consists of a free speech and a mental arithmetic task of 15 min duration performed in front of a panel and a camera (Kirschbaum 1993; Kirschbaum et al 1992a). This stress protocol has been found to induce significant cortisol, ACTH, and cardiovascular responses at the first exposure in 70-80% of all subjects. In a recent meta-analysis, protocols combining uncontrollable and social-evaluative elements - such as the TSST - have been found to produce the largest HPA axis responses (Dickerson and Kemeny 2004).

3.3.3 Blood and Saliva Sampling

Saliva, serum blood and EDTA blood samples were collected 2 min before and 1, 10, 20, 30 and 90 min after cessation of the TSST to assess total cortisol and ACTH levels. Additional saliva samples for the assessment of salivary cortisol were obtained at 45 and 60 min using Salivette sampling devices (Sarstedt, Nuembrecht, Germany). EDTA blood samples were immediately stored on ice and centrifuged within 30 min at 2000 x g and 4° C for 10 min. EDTA plasma was divided into aliquots and stored at -80° C until analysis. Serum blood samples were kept at room temperature for 30 min before they were processed as the plasma samples and stored at -20°C until analysis. Saliva samples were kept at room temperature throughout one test session and then stored at -20°C. After thawing for biochemical analysis, samples were centrifuged at 2000 x g at 10°C for 10 min.

3.3.4 Biochemical Analyses

Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendorfer et al 1992). Total cortisol concentrations were measured in serum blood with an ELISA (IBL, Hamburg, Germany). ACTH was measured in EDTA plasma with a chemiluminescence immunoassay (Nichols institute, Bad Nauheim, Germany). Interassay and intraassay coefficients of variance were below 10% and 12%, respectively, for all analyses.

3.3.5 DNA Extraction and Genotyping

DNA was extracted from 10ml peripheral venous blood following a standard NaCl salting out method according to the protocol of Miller (Miller et al 1988). Genotyping was performed using the allelic discrimination technique, with custom designed primers and probes (Assay by Design service, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands, primer and probe sequences available on request), using TaqMan Universal PCR master mix (Applied Biosystems). Reaction

components and amplification parameters were based on the manufacturer's instructions.

3.3.6 Statistical Analyses

First, all data were log transformed to yield unskewed outcome variables. General Linear Models (GLMs) were computed to assess the repeated measures effect *time*, the between-subjects effect *genotype* as well as the interaction *time x genotype* for endocrine responses to the TSST exposure and the DST. In order to reveal possible sex by genotype interactions, *sex* was also included as predictor in the GLMs. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. All results shown are the mean ± SEM.

In order to allow for appropriate comparison of extreme response genotype groups with a group reflecting the prevalence of the other variants in the population, a comparison group was modelled, which will be referred to as *Representative Comparison* group. This modelling was performed since the over-all group which was phenotyped represents a stratified sample with about equal group sizes not reflecting the actual prevalence in the population (see results). For each comparison with an extreme response group, the respective genotype group were removed from the *Representative Comparison* group. Data on the prevalence of the polymorphisms under investigation were derived from a total of more than 6000 subjects genotyped in the "Rotterdam Study" and from the present study (601 subjects).

3.4 Results

3.4.1 Genotyping

Genotyping of 601 subjects for the four polymorphisms revealed five haplotypes (see Figure 3.1). Allelic frequencies observed correspond to those previously reported (Huizenga et al 1998; Koper et al 1997; van Rossum et al 2006; Van Rossum et al 2004a; van Rossum et al 2002; Van Rossum and Lamberts 2004; Van Rossum et al 2004b; van West et al 2006) and all SNPs were in Hardy Weinberg Equilibrium.

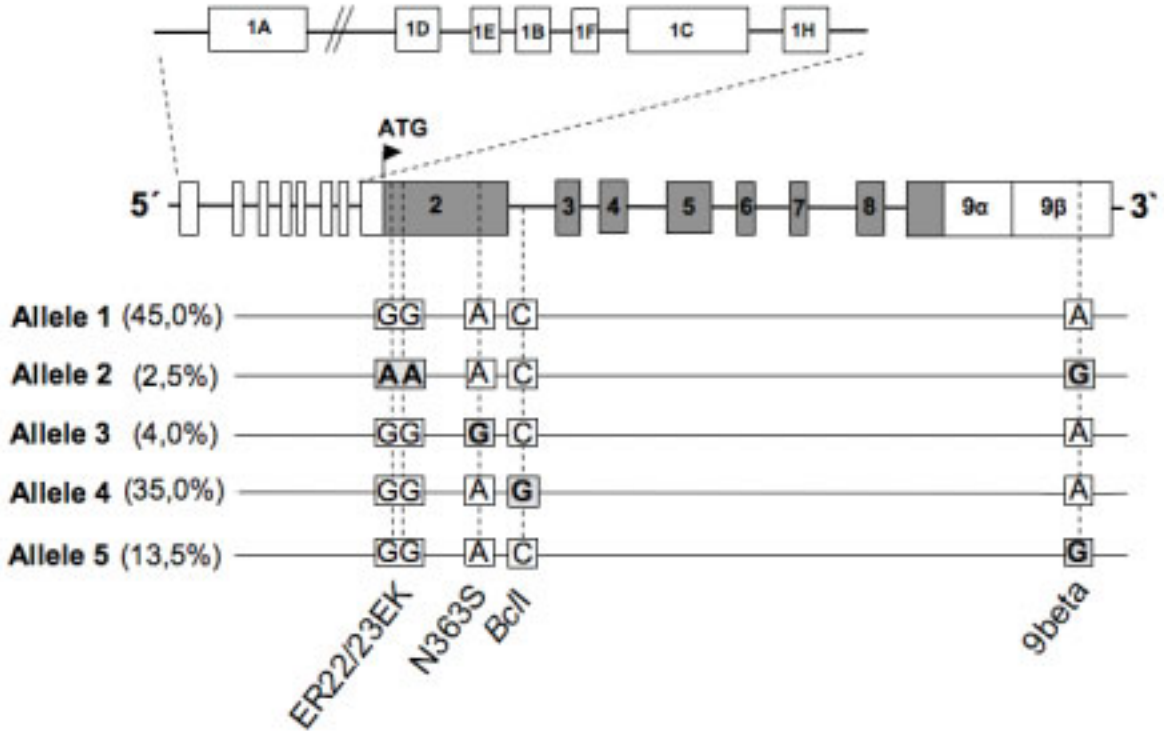

Figure 3.1: *Upper portion of the figure shows genomic organization of the human glucocorticoid receptor gene (NR3C1). Exons are indicated by boxes and the translated part of the gene is shown in darker shade. Lower portion of the figure indicates allelic frequencies of the SNPs and the observed haplotype structure. Base pair substitutions are denoted by bold letters*

The allele with the highest prevalence (45%) will be referred to as the *Most Common Allele* (*MCA*). The GAGAGG to GAAAAG transition at position 22/23 (2.5%) always occurred together with the base change from A to G in exon 9beta, which, in 13.5% of the subjects, was also observed independently from ER22/23EK and the other markers. The base changes from A to G at position 363 (4%) and the intronic change from C to G (*Bcl*I, 35%) also occurred independently of the other investigated variants. Individual haplotype assignments for the four polymorphisms were determined using SNPHAP. Only haplotype assignments with a remaining uncertainty of less than five percent and haplotypes with a frequency over two percent were included in the analyses. Linkage disequilibrium among the four variants was estimated with D' and r2 using Haploview.

The genotype distribution of the 206 phenotyped subjects is indicated in Table 3.1. The intended groups sizes of about 30 subjects per genotype group was achieved for all groups except for the 22/23 variant due to the low minor allele frequency of 2.5%.

Genotype groups	Alleles	N	Women	Men
MCA homozygotes	Allele 1 / Allele 1	31	19	12
*Bcl*I CG	Allele 1 / Allele 4	62	35	27
*Bcl*I GG	Allele 4 / Allele 4	37	24	13
9beta AG	Allele 1 / Allele 5	31	18	13
N363S AG	Allele 3 carriage	27	14	13
ER22/23EK GA	Allele 2 carriage	18	8	10
Total		**206**	**118**	**88**

Table 3.1: *Genotype groups, frequencies and allele combinations. Due to low prevalence of the 9beta G, N363S G and ER22/23EK A alleles, no homozygotes for the respective alleles were investigated. In the 9beta AG group, carriers of the 9beta G allele (allele 5) were only paired with the Most Common Allele (MCA, allele1). In the N363S AG and ER22/23EK group, the variant alleles were paired with allele 1 and allele 4*

3.4.2 TSST

Sex effect

As expected, exposure to the TSST led to significant increases in ACTH, total cortisol and salivary cortisol levels in both men and women (F>60.0, p=.0001 for all analytes; see Figure 3.2 a to c. As previously reported (Kirschbaum et al 1999; Kirschbaum et al 1992b; Kudielka and Kirschbaum 2005), men showed significantly higher increases in ACTH (main effect *sex*: $F_{1,206}$=111.3, p=.0001; interaction *sex* x *time*: $F_{3.1,\ 641.7}$=9.6; p=.0001) and salivary cortisol (main effect *sex*: $F_{1,212}$=14.4, p=.0001; interaction *sex* x *time*: $F_{3.1,\ 649.2}$=26.5, p=.0001), while women, all using OCs, displayed significantly higher levels of total cortisol (main effect *sex*: $F_{1,211}$=37.6 ; p=.0001; interaction *sex* x *time*: $F_{2.6,\ 553.3}$=33.1 ; p=.0001).

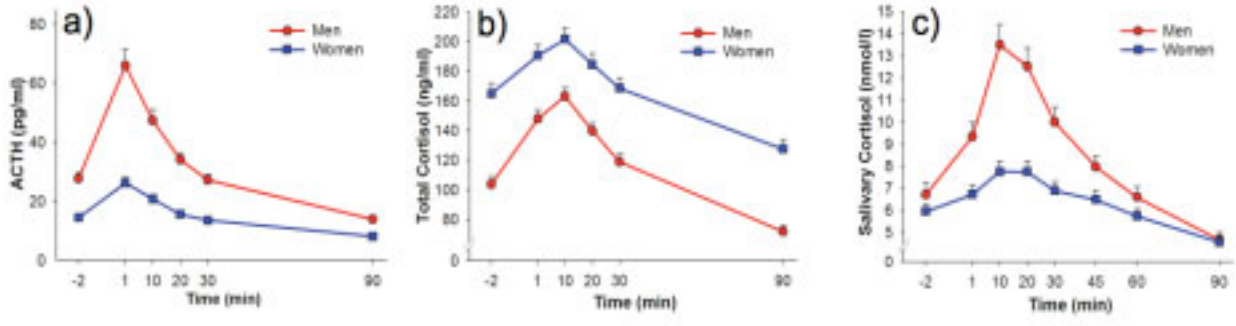

Figure 3.2: *Sex effect in hormonal responses to the TSST. 2a) ACTH 2b) total cortisol and 2c) salivary cortisol levels*

Genotype and genotype by sex effects

For ACTH and cortisol responses following the TSST, large variability between GR genotype groups became evident. ACTH responses in all genotype groups following the TSST are shown in Figure 3.3 a and b for men and women, documenting large variability and at the same time substantial overlap. In order to assess the impact of extreme response groups on the observed variability, the genotype groups with the highest and lowest HPA responses were compared, as shown in Figure 3.3 c. For better illustration, these two extremes are depicted in relation to each other and in relation to the *Representative Comparison* group, which reflects the prevalence of the investigated variants in the population (see Methods).

Male 9beta AG carriers clearly showed the highest ACTH peak levels after TSST exposure and *Bcl*I GG carriers displayed the lowest levels, yielding a significant effect of genotype on ACTH responses (main effect *genotype*: $F_{2,60}$=3.0, p=.05). In women, differences between groups did not yield statistical significance. On a descriptive level, the 9beta AG group was on a relatively high response level, together with the *Most Common Allele* group and the *Bcl*I GG group displayed lower responses, together with the remaining groups (Figure 3.3 b).

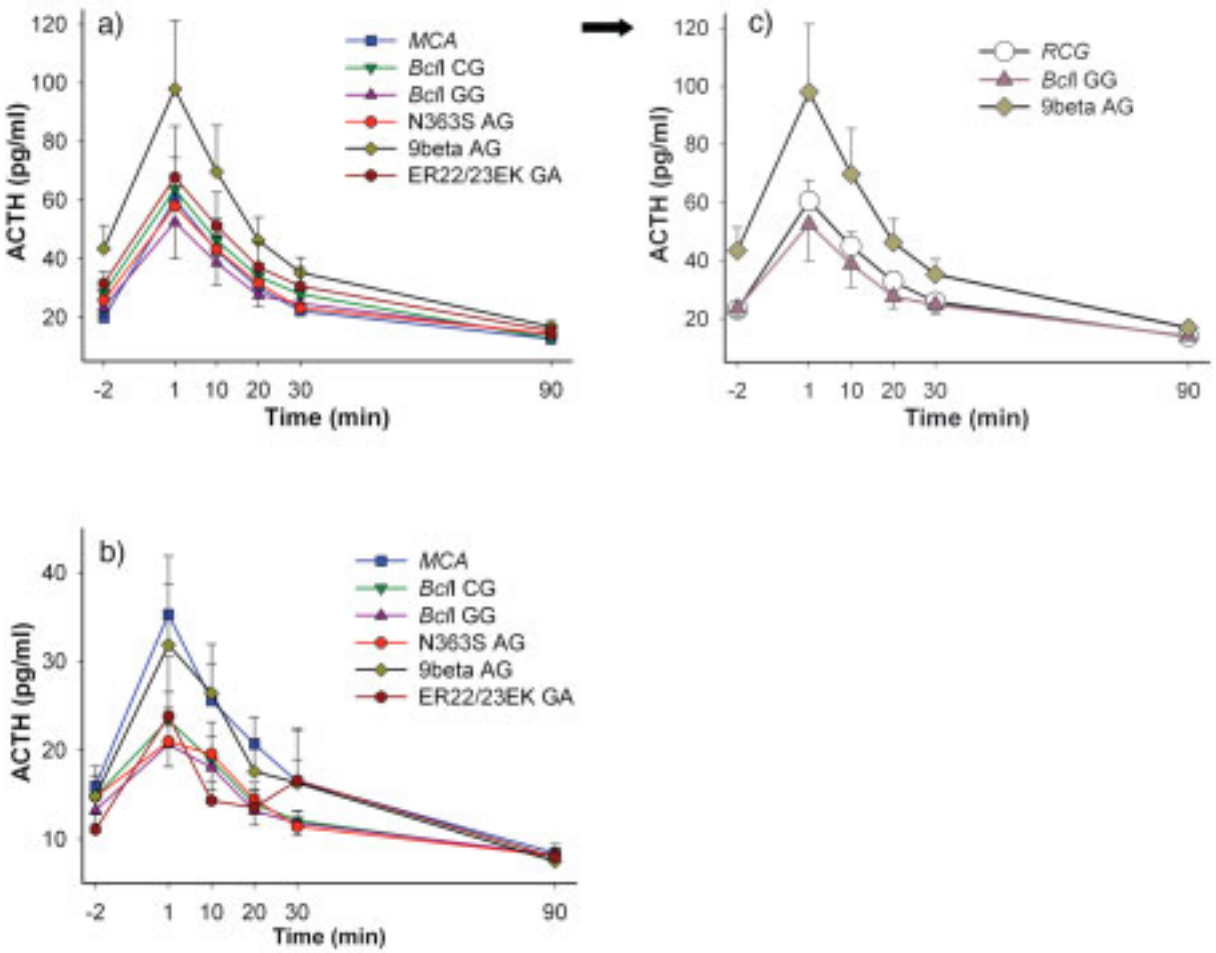

Figure 3.3: *ACTH response following the TSST in a) men for all genotypes and b) women for all genotypes (MCA: Most Common Allele) c) Comparison of extreme response groups (9beta and BclI GG) with the Representative Comparison group (RCG) in men*

Total cortisol responses to the TSST are depicted in Figure 3.4 a and b. Consistent with ACTH levels, male 9beta AG carriers showed the highest and *Bcl*I GG group the lowest levels, yielding a trend towards significance (main effect *genotype*: $F_{2,60}$=1.8, p=.17). In women, no significant group differences were detected. Remarkably, however, the *Bcl*I GG genotype, with lowest total cortisol levels in men, had the highest total cortisol levels in women, resulting in a significant *sex* by *genotype* interaction ($F_{1,112}$=4.6, p=.03), shown in Figure 3.4 c.

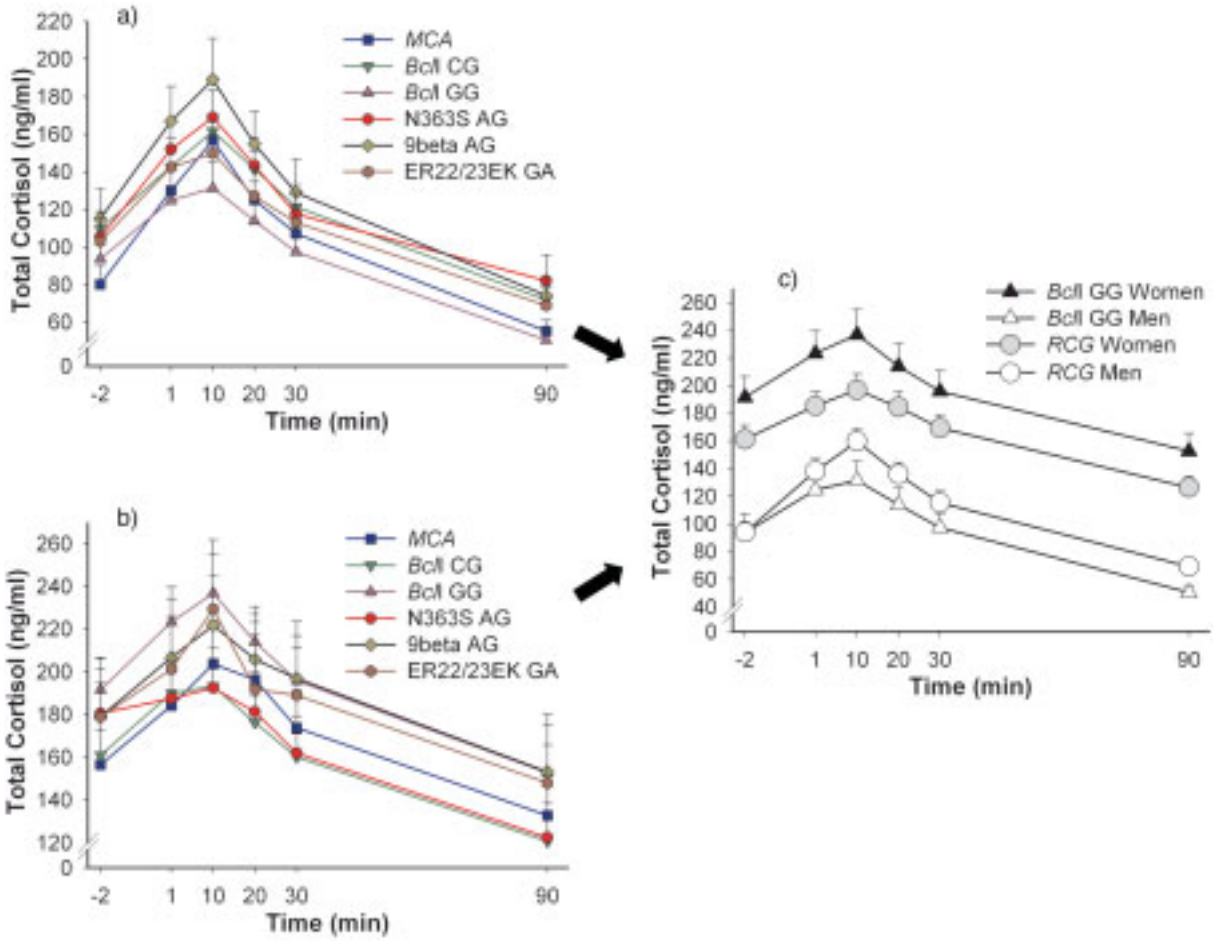

Figure 3.4: *Total cortisol responses following the TSST in a) men for all genotypes and b) women for all genotypes (MCA: Most Common Allele) c) Response curves for the BclI GG genotype in men and women compared with the male and female Representative Comparison group (RCG)*

Salivary cortisol responses to the TSST are shown in Figure 3.5. The lowest salivary cortisol response in men was observed in group *Bcl*I GG while the highest responses were detected for the N363S AG genotype (Figure 3.5 a). In women, all groups except for the N363S AG genotype had very similar response patterns (Figure 3.5 b). Female N363S AG carriers displayed a completely blunted salivary cortisol response and, although not reaching statistical significance (p=.25), the *sex* by *genotype* interaction for the N363S genotype is noteworthy on a descriptive level (Figure 3.5 c).

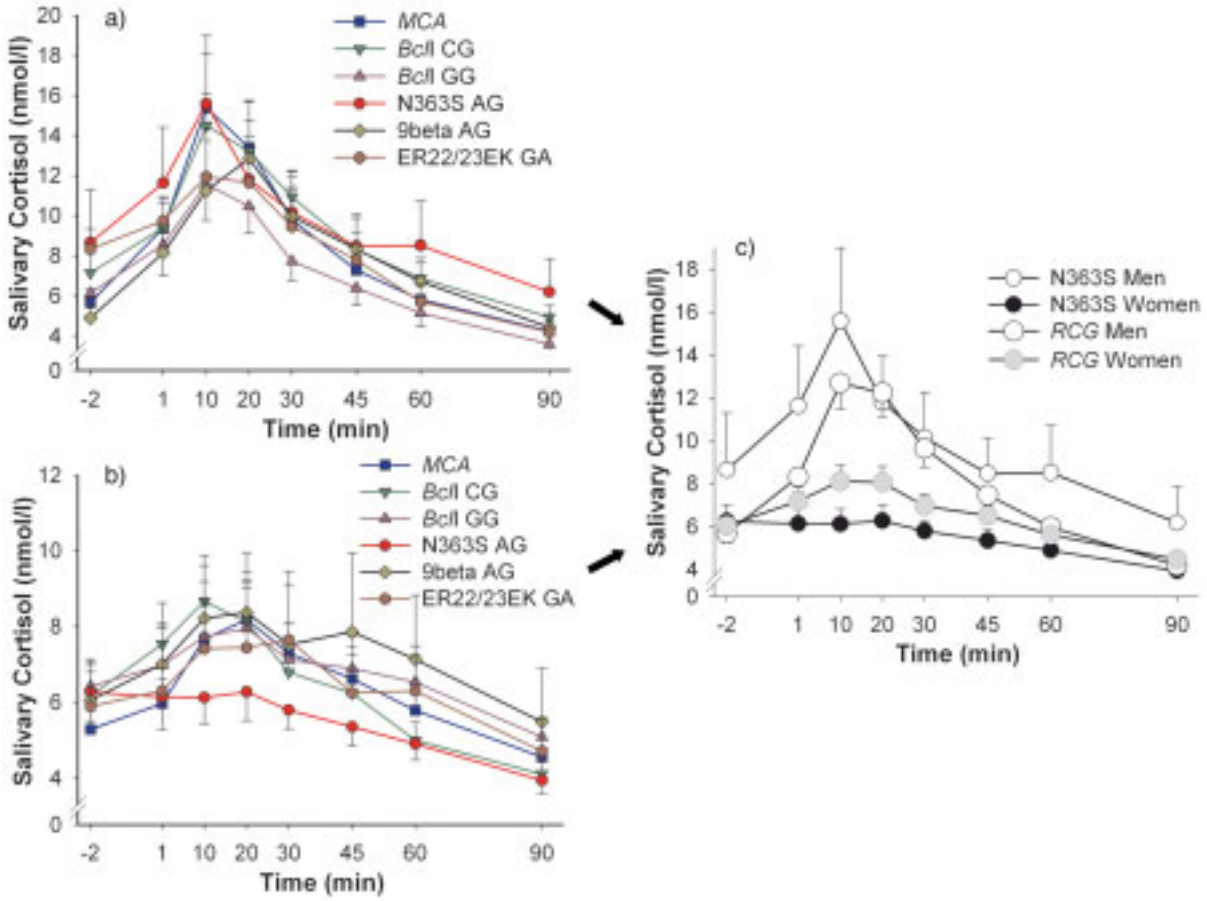

Figure 3.5: *Salivary cortisol responses following the TSST in a) men for all genotypes and b) women for all genotypes (MCA: Most Common Allele) c) Response curves for the N363S AG genotype in men and women compared with the male and female Representative Comparison group (RCG)*

3.5 Discussion

Variations of the GR gene can modulate the endocrine stress response and possibly represent one factor underlying vulnerability for psychopathology. Therefore, the present study investigated all GR gene polymorphisms, which, at present time, seem to be of functional relevance for the regulation of the HPA axis. The comparison of extreme response groups revealed significant associations between GR gene variants and HPA axis responses to psychosocial stress and measures. In addition, for the first time, marked sex by genotype interactions were observed for three GR gene variants. Male carriers of the 9beta AG variant displayed enhanced ACTH and serum cortisol responses following psychosocial stress, while male *Bcl*I GG carriers showed relatively diminished ACTH, serum and salivary cortisol levels. In women (all using oral contraceptives; see below for discussion), conversely, *Bcl*I GG carriers showed highest serum cortisol levels in response to the TSST. On a descriptive level,

remarkable response patterns for salivary cortisol following TSST were observed for the N363S genotype. While male 363S carriers had the highest responses, no significant increase became evident for women.

A marked sexual dimorphism is a well-documented determinant of interindividual variability of HPA axis regulation. Different HPA axis response patterns in males and females have been observed in animal as well as in human studies (Kajantie and Phillips 2006; Kirschbaum et al 1999; Kitay 1963; Roelfsema et al 1993; Uhart et al 2006). One of the factors possibly mediating these sex differences is the level of circulating gonadal steroids (Burgess and Handa 1992; Carey et al 1995; Norman et al 1992; Viau and Meaney 1991). The observed sex by genotype interactions in our study suggest that the same genetic variant of the GR gene can have differential, even opposite effects on HPA axis responses. In a recent publication, Weiss and colleagues (2006) noted that the cellular environment in women and men differs substantially regarding the hormonal milieu and they highlighted the impact of sex on the penetrance and expressivity of a wide variety of traits. The differences in hormonal levels might lead to differential expressivity of the underlying genetic networks, thus, gene by 'cellular environment' interactions can result in differential effects of the same variation in men and women. A sex specific genetic architecture of quantitative traits has been observed for a number of measures (Korstanje et al 2004; Solberg et al 2004; Stone et al 2004; Wang et al 2006; Weiss et al 2005a; Weiss et al 2005b; Williams et al 2003), including basal morning cortisol levels (Kurina et al 2005), supporting the notion of sex specific associations of genes involved in HPA axis regulation. In light of the existing sexual dimorphism in prevalence of disease in general (men are more susceptible to cardiovascular and infectious disease, women develop major depression, autoimmune disease and chronic pain more often), it has been hypothesized that sex differences in the physiological stress response may at least in part explain these differences in susceptibility to disease (Kajantie and Phillips 2006; Kudielka and Kirschbaum 2005). Our results suggest, in turn, that genetic factors might differentially influence determinants of stress responsivity. It has to be noted that all female participants of our study were using ethinyl-estradiol containing oral contraceptives. We chose to investigate only OC using women in order to avoid modulation of hormonal responses to the TSST by the menstrual cycle phase. It is well known that ethinyl-estradiol leads to increases in corticosteroid binding globulin (CBG) synthesis, which in turn influences total serum cortisol levels and the availability of free cortisol.

Indeed, CBG was shown to modulate hormonal responses to TSST exposure in OC using females, as well as in males (Kumsta, unpublished). Since all our female subjects were using OCs, a generalization of our findings on women not taking OCs is not possible. However, OC intake resulting in high levels of CBG are most likely not responsible for our observed genotype by sex interactions, since all women were taking OCs and in addition, we did not observe significantly different CBG levels between the genotype groups (p=.30). However, it certainly cannot be ruled out that other unknown consequences of OC intake – related to or independent of CBG – impact on our measure in a genotype specific way.

The strongest effects for hormonal responses following the TSST were observed for 9beta AG and *Bcl*I GG carriers in men. It has been demonstrated that the 9beta variant increases GRbeta mRNA stability, possibly leading to higher expression of GRbeta protein (Derijk et al 2001; Schaaf and Cidlowski 2002a), which serves as a dominant negative inhibitor of GRalpha (Charmandari et al 2005; Oakley et al 1999; Schaaf and Cidlowski 2002b; Yudt et al 2003). Furthermore, a lower transrepressing activity has been reported for this variant (van den Akker et al 2006b) and a recent study showed a lower risk of persistent *Staphylococcus Aureus* nasal carriage in 9beta GG carriers, possibly resulting from reduced GC induced immune suppression (van den Akker et al 2006a). Our data suggest a relative GC insensitivity in 9beta AG carriers as indicated by enhanced ACTH responses following the TSST. These observations are in accordance with the functional analyses and the observed associations with rheumatoid arthritis (Derijk et al 2001) and *Staphylococcus Aureus* carriage. However, very low levels of GRbeta mRNA and protein have been observed in hippocampus (DeRijk et al 2003) and other tissues (Pujols et al 2002) so that the question remains how such small quantities of GRbeta protein can yield the observed effects. Expression of GRbeta mRNA and protein have not been tested in the pituitary, the major site where Dexamethasone exerts its effects, and warrants further investigations. The factor necessary for alternative splicing of the GR pre-mRNA was identified as SRp30c (serine-arginine-rich protein 30c; Xu et al 2003). Different cell types show varying levels of GRbeta, which might be due to different levels of SRp30c expression. Treatment of neutrophils with IL-8 leads to enhanced expression of both SRp30c and GRbeta. Furthermore, inflammatory processes (Webster et al 2001) increase the abundance of GRbeta mRNA and protein. These data indicate that under certain conditions levels of GRbeta protein can reach functionally relevant levels.

For the intronic *Bcl*I polymorphism, no data on putative functional mechanisms are available. However, this variant has been associated with measures of body composition, metabolic parameters and sensitivity towards GC in previous studies (Van Rossum and Lamberts 2004). Decreased HPA axis responses following psychosocial stress were observed in *Bcl*I GG carriers in men (Wüst et al 2004) and this finding could be replicated in the present study. Furthermore, the *Bcl*I GG genotype was associated with an increased susceptibility to develop major depression (van Rossum et al 2006). One could speculate that increased sensitivity to GC in the brain would lead to enhanced negative feedback on hypothalamic and pituitary level and at the same time to an increased CRF expression in the limbic system (Reul and Holsboer 2002; Schulkin et al 1998), which would explain the higher vulnerability for depression. The question remains, however, why female *Bcl*I GG carriers show the highest serum cortisol responses, concomitantly with the lowest ACTH levels. This observation might reflect an increased adrenal sensitivity to ACTH although no differences in adrenal sensitivity following 1µg ACTH stimulation were observed between genotype groups (data not shown). Possibly, the low dose of 1µg is still too high to discern differences in adrenal sensitivity.

For the N363S genotype, the distinctly elevated cortisol levels following TSST in men could not be observed as clearly as in our previous study (Wüst et al 2004). However, male 363S carriers descriptively still showed the highest responses in salivary cortisol to psychosocial stress.

One limitation of the present study is the small sample size and the accompanying problem of limited statistical power to detect associations. Our strategy of intensive phenotyping, together with low prevalence of some the investigated variants, inevitably leads to small group sizes. Given these limitations, it is remarkable that relatively clear and rather consistent results in the different test and in two independent studies could be observed. Possibly, the experimental nature of our design can partly compensate for the relatively modest sample size. The present investigation can be considered as a ‘neuroscience based experimental candidate gene study’ (Caspi and Moffitt 2006), which focused on relevant genetic polymorphisms, employed valid neuroendocrine challenge paradigms and assessed continuous variables instead of dichotomic data (e.g. ‘affected’ vs. ‘non affected’).

In summary, we investigated the associations between HPA axis responses to challenge and all known and, at present state of research, functionally relevant genetic polymorphisms of the glucocorticoid receptor with significant prevalence in

the general population. Our approach to study all known relevant GR gene polymorphisms simultaneously in an extensively characterized sample provides first insight on the proportion of the marked variability in HPA axis responses to psychosocial stress that can be attributed to genetic variability of the GR. In addition, we observed for the first time in a thoroughly phenotyped sample evidence for sex specific effects of GR polymorphisms on HPA axis activity. Both human (Holsboer 2000; Neigh and Nemeroff 2006; Pariante and Miller 2001) and animal studies (Howell and Muglia 2006; Ridder et al 2005) have implicated changes in GR function in the etiology of depression or depression-like behaviors. It is likely that polymorphisms in the GR, which can impact on the efficacy of GC signaling and profoundly influence the downstream biology of peripheral and central GC responsive systems, can significantly contribute to an increased or decreased susceptibility to mental disease.

3.6 References

van den Akker EL, Nouwen JL, Melles DC, Rossum EF, Koper JW, Uitterlinden AG, et al (2006a): Staphylococcus aureus Nasal Carriage Is Associated with Glucocorticoid Receptor Gene Polymorphisms. *J Infect Dis* 194:814-818.

van den Akker EL, Russcher H, van Rossum EF, Brinkmann AO, de Jong FH, Hokken A, et al (2006b): Glucocorticoid receptor polymorphism affects transrepression but not transactivation. *J Clin Endocrinol Metab* 91:2800-2803.

Bamberger CM, Bamberger AM, de Castro M, Chrousos GP (1995): Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest* 95:2435-2441.

Bamberger CM, Schulte HM, Chrousos GP (1996): Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 17:245-261.

Bjorntorp P, Rosmond R (2000): The metabolic syndrome--a neuroendocrine disorder? *Br J Nutr* 83 Suppl 1:S49-57.

Burgess LH, Handa RJ (1992): Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* 131:1261-1269.

Carey MP, Deterd CH, de Koning J, Helmerhorst F, de Kloet ER (1995): The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. *J Endocrinol* 144:311-321.

Caspi A, Moffitt TE (2006): Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci* 7:583-590.

Charmandari E, Chrousos GP, Ichijo T, Bhattacharyya N, Vottero A, Souvatzoglou E, Kino T (2005): The human glucocorticoid receptor (hGR) beta isoform suppresses the transcriptional activity of hGRalpha by interfering with formation of active coactivator complexes. *Mol Endocrinol* 19:52-64.

Di Blasio AM, van Rossum EF, Maestrini S, Berselli ME, Tagliaferri M, Podesta F, et al (2003): The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients. *Clin Endocrinol (Oxf)* 59:68-74.

Dickerson SS, Kemeny ME (2004): Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychol Bull* 130:355-391.

Dobson MG, Redfern CP, Unwin N, Weaver JU (2001): The N363S polymorphism of the glucocorticoid receptor: potential contribution to central obesity in men and lack of association with other risk factors for coronary heart disease and diabetes mellitus. *J Clin Endocrinol Metab* 86:2270-2274.

Dressendorfer RA, Kirschbaum C, Rohde W, Stahl F, Strasburger CJ (1992): Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. *J Steroid Biochem Mol Biol* 43:683-692.

Echwald SM, Sorensen TI, Andersen T, Pedersen O (2001): The Asn363Ser variant of the glucocorticoid receptor gene is not associated with obesity or weight gain in Danish men. *Int J Obes Relat Metab Disord* 25:1563-1565.

Fleury I, Beaulieu P, Primeau M, Labuda D, Sinnett D, Krajinovic M (2003): Characterization of the *Bcl*I polymorphism in the glucocorticoid receptor gene. *Clin Chem* 49:1528-1531.

Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ, et al (1999): Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 159:1600-1604.

Holsboer F (2000): The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* 23:477-501.

Holsboer F (2001): Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J Affect Disord* 62:77-91.

Howell MP, Muglia LJ (2006): Effects of genetically altered brain glucocorticoid receptor action on behavior and adrenal axis regulation in mice. *Front Neuroendocrinol*.

Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, et al (1998): A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *J Clin Endocrinol Metab* 83:144-151.

Jacobson L, Sapolsky R (1991): The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews* 12:118-134.

Kajantie E, Phillips DI (2006): The effects of sex and hormonal status on the physiological response to acute psychosocial stress. *Psychoneuroendocrinology* 31:151-178.

Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH (1999): Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med* 61:154-162.

Kirschbaum C, Pirke, K. M. & Hellhammer, D. H. (1993): The 'Trier Social Stress Test' - a tool for investigating psychobiology stress responses in a laboratory setting. *Neuropsychobiology* 28:76-81.

Kirschbaum C, Wüst S, Faig HG, Hellhammer DH (1992a): Heritability of cortisol responses to human corticotropin-releasing hormone, ergometry, and psychological stress in humans. *J Clin Endocrinol Metab* 75:1526-1530.

Kirschbaum C, Wust S, Hellhammer D (1992b): Consistent sex differences in cortisol responses to psychological stress. *Psychosom Med* 54:648-657.

Kitay JI (1963): Pituitary-Adrenal Function in the Rat after Gonadectomy and Gonadal Hormone Replacement. *Endocrinology* 73:253-260.

de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475.

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, et al (1997): Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. *Hum Genet* 99:663-668.

Korstanje R, Li R, Howard T, Kelmenson P, Marshall J, Paigen B, Churchill G (2004): Influence of sex and diet on quantitative trait loci for HDL cholesterol levels in an SM/J by NZB/BINJ intercross population. *J Lipid Res* 45:881-888.

Kudielka BM, Kirschbaum C (2005): Sex differences in HPA axis responses to stress: a review. *Biol Psychol* 69:113-132.

Kurina LM, Weiss LA, Graves SW, Parry R, Williams GH, Abney M, Ober C (2005): Sex differences in the genetic basis of morning serum cortisol levels: genome-wide screen identifies two novel loci specific to women. *J Clin Endocrinol Metab* 90:4747-4752.

Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, et al (1997): Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* 186:1567-1574.

Lin RC, Wang WY, Morris BJ (1999): High penetrance, overweight, and glucocorticoid receptor variant: case-control study. *Bmj* 319:1337-1338.

Lin RC, Wang XL, Dalziel B, Caterson ID, Morris BJ (2003): Association of obesity, but not diabetes or hypertension, with glucocorticoid receptor N363S variant. *Obes Res* 11:802-808.

Miller SA, Dykes DD, Polesky HF (1988): A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.

Neigh GN, Nemeroff CB (2006): Reduced glucocorticoid receptors: consequence or cause of depression? *Trends Endocrinol Metab* 17:124-125.

Norman RL, Smith CJ, Pappas JD, Hall J (1992): Exposure to ovarian steroids elicits a female pattern of plasma cortisol levels in castrated male macaques. *Steroids* 57:37-43.

Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA (1999): The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem* 274:27857-27866.

Pariante CM, Miller AH (2001): Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* 49:391-404.

Parker AJ, Wessely S, Cleare AJ (2001): The neuroendocrinology of chronic fatigue syndrome and fibromyalgia. *Psychol Med* 31:1331-1345.

Pujols L, Mullol J, Roca-Ferrer J, Torrego A, Xaubet A, Cidlowski JA, Picado C (2002): Expression of glucocorticoid receptor alpha- and beta-isoforms in human cells and tissues. *Am J Physiol Cell Physiol* 283:C1324-1331.

Reichardt HM, Schutz G (1998): Glucocorticoid signalling--multiple variations of a common theme. *Mol Cell Endocrinol* 146:1-6.

Reul JM, Holsboer F (2002): Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* 2:23-33.

Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, et al (2005): Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J Neurosci* 25:6243-6250.

de Rijk RH, Schaaf M, Stam FJ, de Jong IE, Swaab DF, Ravid R, et al (2003): Very low levels of the glucocorticoid receptor beta isoform in the human hippocampus as shown by Taqman RT-PCR and immunocytochemistry. *Brain Res Mol Brain Res* 116:17-26.

de Rijk RH, Schaaf MJ, Turner G, Datson NA, Vreugdenhil E, Cidlowski J, et al (2001): A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol* 28:2383-2388.

Roelfsema F, van den Berg G, Frolich M, Veldhuis JD, van Eijk A, Buurman MM, Etman BH (1993): Sex-dependent alteration in cortisol response to endogenous adrenocorticotropin. *J Clin Endocrinol Metab* 77:234-240.

Rosmond R, Chagnon YC, Holm G, Chagnon M, Perusse L, Lindell K, et al (2000): A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. *Obes Res* 8:211-218.

van Rossum EF, Binder EB, Majer M, Koper JW, Ising M, Modell S, et al (2006): Polymorphisms of the glucocorticoid receptor gene and major depression. *Biol Psychiatry* 59:681-688.

van Rossum EF, Feelders RA, Van Den Beld AW, Uitterlinden AG, Janssen JA, Ester W, et al (2004a): Association of the ER22/23EK polymorphism in the glucocorticoid receptor gene with survival and C-reactive protein levels in elderly men. *Am J Med* 117:158-162.

van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, et al (2002): A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 51:3128-3134.

van Rossum EF, Koper JW, Van Den Beld AW, Uitterlinden AG, Arp P, Ester W, et al (2003): Identification of the *Bcl*I polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. *Clin Endocrinol (Oxf)* 59:585-592.

van Rossum EF, Lamberts SW (2004): Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. *Recent Prog Horm Res* 59:333-357.

van Rossum EF, Voorhoeve PG, Te Velde SJ, Koper JW, Delemarre-Van De Waal HA, Kemper HC, Lamberts SW (2004b): The ER22/23EK Polymorphism in the Glucocorticoid Receptor Gene Is Associated with a Beneficial Body Composition and Muscle Strength in Young Adults. *J Clin Endocrinol Metab* 89:4004-4009.

Russcher H, Smit P, van den Akker EL, van Rossum EF, Brinkmann AO, de Jong FH, et al (2005a): Two Polymorphisms in the Glucocorticoid Receptor Gene Directly Affect Glucocorticoid-Regulated Gene Expression. *J Clin Endocrinol Metab*.

Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW (2005b): Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. *Mol Endocrinol* 19:1687-1696.

Schaaf MJ, Cidlowski JA (2002a): AUUUA motifs in the 3'UTR of human glucocorticoid receptor alpha and beta mRNA destabilize mRNA and decrease receptor protein expression. *Steroids* 67:627-636.

Schaaf MJ, Cidlowski JA (2002b): Molecular mechanisms of glucocorticoid action and resistance. *J Steroid Biochem Mol Biol* 83:37-48.

Schulkin J, Gold PW, McEwen BS (1998): Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. *Psychoneuroendocrinology* 23:219-243.

Solberg LC, Baum AE, Ahmadiyeh N, Shimomura K, Li R, Turek FW, et al (2004): Sex- and lineage-specific inheritance of depression-like behavior in the rat. *Mamm Genome* 15:648-662.

Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ, Lee TH (2000): Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol* 105:943-950.

Stone JL, Merriman B, Cantor RM, Yonan AL, Gilliam TC, Geschwind DH, Nelson SF (2004): Evidence for sex-specific risk alleles in autism spectrum disorder. *Am J Hum Genet* 75:1117-1123.

Uhart M, Chong RY, Oswald L, Lin PI, Wand GS (2006): Gender differences in hypothalamic-pituitary-adrenal (HPA) axis reactivity. *Psychoneuroendocrinology* 31:642-652.

Viau V, Meaney MJ (1991): Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* 129:2503-2511.

Wang S, Yehya N, Schadt EE, Wang H, Drake TA, Lusis AJ (2006): Genetic and Genomic Analysis of a Fat Mass Trait with Complex Inheritance Reveals Marked Sex Specificity. *PLoS Genetics* 2:e15.

Webster JC, Oakley RH, Jewell CM, Cidlowski JA (2001): Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. *Proc Natl Acad Sci U S A* 98:6865-6870.

Weiss LA, Abney M, Cook EH, Jr., Ober C (2005a): Sex-specific genetic architecture of whole blood serotonin levels. *Am J Hum Genet* 76:33-41.

Weiss LA, Abney M, Parry R, Scanu AM, Cook EH, Jr., Ober C (2005b): Variation in ITGB3 has sex-specific associations with plasma lipoprotein(a) and whole blood serotonin levels in a population-based sample. *Hum Genet* 117:81-87.

Weiss LA, Pan L, Abney M, Ober C (2006): The sex-specific genetic architecture of quantitative traits in humans. *Nat Genet* 38:218-222.

van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, et al (2006): Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. *Neuropsychopharmacology* 31:620-627.

Williams RB, Marchuk DA, Gadde KM, Barefoot JC, Grichnik K, Helms MJ, et al (2003): Serotonin-related gene polymorphisms and central nervous system serotonin function. *Neuropsychopharmacology* 28:533-541.

Wüst S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH (2004): Common Polymorphisms in the Glucocorticoid Receptor Gene Are Associated with Adrenocortical Responses to Psychosocial Stress. *J Clin Endocrinol Metab* 89:565-573.

Xu Q, Leung DY, Kisich KO (2003): Serine-arginine-rich protein p30 directs alternative splicing of glucocorticoid receptor pre-mRNA to glucocorticoid receptor beta in neutrophils. *J Biol Chem* 278:27112-27118.

Yehuda R (1997): Sensitization of the hypothalamic-pituitary-adrenal axis in posttraumatic stress disorder. *Ann N Y Acad Sci* 821:57-75.

Yudt MR, Jewell CM, Bienstock RJ, Cidlowski JA (2003): Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* 23:4319-4330.

CHAPTER 4

Association of Common Glucocorticoid Receptor Gene Variants with Sensitivity to Glucocorticoids in Different Tissues

4.1 Summary

Background: A considerable variability in the sensitivity to glucocorticoids (GCs) exists between individuals. Variations of the glucocorticoid receptor (GR) gene are thought to account for part of the differences in GC responsiveness.

Methods: We assessed the association between four common GR gene polymorphisms (ER22/23EK, N363S, *Bcl*I, 9beta) and markers of GC sensitivity in three target tissues in 206 healthy individuals. GC sensitivity of subdermal blood vessels was estimated with a skin vasoconstriction assay using beclomethasone. A low dose (0.25 mg) Dexamethasone (Dex) suppression test was used to assess GC sensitivity on pituitary level and the inhibition of LPS-stimulated cytokine production by leukocytes after coincubation with increasing levels of Dex provided a marker of GC sensitivity of circulating leukocytes.

Results: Following the Dex suppression test, male 9beta AG carriers displayed a relative non-suppression of ACTH while no differences between genotypes were observed in women, resulting in a significant *sex* by *genotype* interaction (p=.03). 9beta AG men also showed the highest increase and the highest peak levels in post Dexamethasone salivary cortisol levels in the morning (main effect *genotype*: p=.05). The *Bcl*I GG genotype group showed the least degree of skin blanching, reflecting a lower GC sensitivity of subdermal blood vessels (main effect *genotype*: p=.01). No association between GR genotype and GC sensitivity of peripheral leukocytes was observed.

Conclusions: Common polymorphisms of the GR gene were associated with some measures of GC sensitivity in a sex and tissue specific manner. Variants of the GR gene might be implicated in the development of diseases related to altered GC production and sensitivity.

4.2 Introduction

Glucocorticoids (GCs) are a vital class of steroid hormones with important regulatory roles throughout the organism. Secreted by the adrenal cortex, levels of circulating GCs are regulated through hypothalamus-pituitary-adrenal (HPA) axis activity. GCs have important functions in development, energy metabolism and behavior and they impact on a number of physiological systems, including the immune system and the HPA axis itself (Munck et al 1984). Glucocorticoids also represent very commonly prescribed drugs, routinely used for their antiinflammatory or immune-suppressive effects in patients with chronic inflammatory or autoimmune diseases (DeRijk and Sternberg 1997; Lamberts et al 1996). Within the normal population, a considerable variability in the sensitivity to glucocorticoids across individuals has been observed (Baxter and Rousseau 1979; Huizenga et al 1998b). Furthermore, it has been shown that the GC sensitivity of one target tissue does not reflect the GC sensitivity of other organs in patients receiving GC treatment (Corrigan et al 1991; Corrigan et al 1996; Sher et al 1994) and in healthy individuals (Ebrecht et al 2000; Vasiliadi et al 2002). GCs work mainly through the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. The GR mediates transactivation or -repression of GC responsive genes by direct DNA binding or by protein-protein interactions with other transcription factors. The magnitude and efficacy of GC action depends, besides other factors, on characteristics of the GR. It has been hypothesized that genetic variations of the GR are associated with the observed variability in GC responsiveness. Clinical abnormalities in GC sensitivity, such as the rare generalized inherited GC resistance syndrome, have been linked to mutations of the GR (for review see Charmandari et al 2004). More common polymorphisms of the GR gene, namely the ER22/23EK, N363S and the intronic *Bcl*I, have been associated with variability in sensitivity to exogenous GCs in the general population.

The ER22/23EK polymorphism (rs6189 & rs6190), located in exon 2, consists of two linked SNPs in codon 22 and 23 separated by 1bp (Koper et al 1997). The base change in codon 22 is silent with both GAG and GAA coding for glutamic acid, whereas the change from AGG to AAG in codon 23 leads to an amino acid change from arginine (R) to lysine (K). A reduced sensitivity in a Dexamethasone (Dex) suppression test was shown for 22/23EK carriers in response to 1mg of Dex but not

a lower dose of 0.25 mg (van Rossum et al 2002). Functional analyses revealed that the ER22/23EK polymorphism leads to a higher expression of the translational GR-A isoform at the expense of the GR-B, resulting in a reduced transactivating capacity of the 22/23EK allele (Russcher et al 2005a).

The N363S (rs 6195) variant is also located in exon 2 and leads to a non-synonymous amino acid change from asparagine (N) to serine (S) in codon 363. *In vivo*, this polymorphism appeared to be associated with increased sensitivity to GCs as indicated by enhanced cortisol suppression and increased insulin secretion following 1mg Dex administration (Huizenga et al 1998a; Koper et al 1997). No differences in concentrations of Dex necessary to achieve half maximal inhibition (IC_{50}) in a mitogen-induced proliferation assay were found between 363S and 363N carriers (Huizenga et al 1998a) and no association in post Dex (0.5 mg) ACTH levels were revealed fort the N363S genotype in males (Wüst et al 2004). Functional *in vitro* studies showed a higher efficiency of the 363S allele in transactivating activity, but not in transrepression, compared to the 363N allele (Russcher et al 2005a).

The *Bcl*I polymorphism (no rs number assigned) in intron B (Fleury et al 2003; Van Rossum et al 2003) was also shown to be associated with measures of GC sensitivity. Van Rossum et al (2003) found decreased cortisol levels following Dex suppression at dosages of 1 mg as well as at 0.25 mg in *Bcl*I G carriers. Stevens et al. (2004) found a three-marker haplotype across intron B, including the *Bcl*I G allele, to be associated with decreased post Dex (0.25 mg) cortisol levels. GC sensitivity on subdermal blood vessels was assessed with the synthetic GC budenosid and revealed reduced skin vasoconstriction after application of budenoside in *Bcl*I G homozygotes (Panarelli et al 1998), indicating tissue specific associations between this variant and GC sensitivity.

Another common variant of the GR (rs 6198) is an A to G transition at position 3669 in the 3´untranslated region (UTR) in exon 9beta. The G allele has been associated with a reduced sensitivity to GCs; however, no studies using exogenous GC administration have been performed. Functional studies revealed a stabilizing effect of this polymorphism on GRbeta mRNA *in vitro*, possibly leading to enhanced expression of GRbeta protein (Derijk et al 2001). GRbeta protein acts as a dominant negative inhibitor of GRalpha (Charmandari et al 2005; Oakley et al 1999; Yudt et al 2003), and enhanced expression of the GRbeta was associated with GC resistance in allergic disease (Hamid et al 1999; Leung et al 1997; Sousa et al 2000). A study with rheumatoid arthritis patients showed a higher prevalence of the 9beta variant in

patients than in controls (Derijk et al 2001) and a lower transrepressing activity has been reported for the G allele (van den Akker et al 2006b).

The aim of the present study was to determine the association between four common GR gene polymorphisms (ER22/23EK, N363S, *Bcl*I and 9beta) and sensitivity to GCs of three target tissues. On blood vessels, GCs and mineralocorticoids interact with vascular receptors and indirectly influence vascular tone by increasing vascular sensitivity to noradrenaline. In the immune system, GCs inhibit the release of the proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) from monocytes and macrophages. On the level of the pituitary, GCs exert a strong feedback signal controlling HPA axis activity. We assessed 1) the intensity of skin blanching in response to topically applied GCs as a marker of GC sensitivity of subdermal blood vessels 2) the inhibition of LPS-stimulated proinflammotary cytokine production by leukocytes after coincubation with increasing levels of Dex, providing a marker of GC sensitivity of circulating leukocytes, and 3) the degree of ACTH and salivary cortisol suppression in response to 0.25 mg Dex, reflecting GC sensitivity on level of the pituitary. Furthermore, we investigated if the intercorrelation in GC sensitivity between the target tissues studied is GR genotype specific.

4.3 Methods

4.3.1 Subjects and Study Design

An initial sample of 601 healthy subjects was recruited from a Trier-based community sample and from students of the University of Trier. These subjects were genotyped for the GR variants under investigation. Subsequently, 206 subjects were selected according to their GR genotype, which resulted in a stratified sample of about equally sized comparison groups (see Results). The actually phenotyped study sample consisted of 118 females and 88 males (mean age 25.1 with standard error of mean (SEM) of ± 3.9 yrs, BMI 24.2 ± 4.1). Participants were non-smokers and of central European descent. Only females using ethinyl-estradiol containing oral contraceptives (OC) were included in order to avoid potential modulation of cortisol levels following Dex suppression by menstrual cycle phase. Except for the use of OC, all subjects reported to be medication free. Before the first experimental session, the absence of acute or chronic diseases was confirmed in a medical exam. The protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants.

Participants of the initial sample reported to our laboratory for collection of a blood sample for later genotype analysis. Subjects were selected according to GR genotype for further testing. For experiments on GC sensitivity, subjects reported to the laboratory three times. On the first day, a blood sample was drawn between 1400 and 1500h to assess sensitivity in peripheral leukocytes. Thereafter, skin blanching was induced and the degree of skin blanching was rated the following day. Subjects then received a tablet containing 0.25 mg of Dex and reported the day after Dex ingestion for collection of a blood sample.

4.3.2 Dexamethasone Suppression Test (DST)

Participants were instructed to ingest 0.25 mg Dex (Par Pharmaceutical, Spring Valley, USA) at 2300 h, and they reported to the laboratory the next morning between 0800 and 0830 h, i.e. 60 to 90 min after awakening, for collection of a blood sample for analysis of ACTH. In addition, subjects were instructed to collect saliva samples (Salivette, Sarstedt, Nuembrecht, Germany) at awakening and 30, 45 and 60 min thereafter (Cortisol awakening response, CAR) on the morning after Dex ingestion. In addition, saliva samples were collected at 0800, 1100, 1500 and 2000h. Upon return, saliva samples were stored at -20°C. After thawing for biochemical analysis, samples were centrifuged at 2000 x g at 10°C for 10 min.

4.3.3 Skin Vasoconstriction Assay

Solutions of beclomethasone dipropionate (Sigma) were prepared in ethanol/water (95:5, vol/vol) at concentrations of 0, 0.2, 1, 5, 10, and 20 µg/ml. Six circles with a 25-mm diameter were outlined on the volar aspect of the subject's forearm. 50 µl of each solution were applied to a corresponding circle between 1600 and 1700 h in randomized order. After evaporation of ethanol, the forearm was covered with polyethylene vacuum foil. The occlusive dressing was removed the following day between 1400 and 1500 h. Thirty min later, the intensity of the skin blanching was rated for each circle. The test areas were examined by two trained, blinded raters under standardized light conditions. Scores on a standardized rating scale ranged from 0 (no blanching), 1 (faint blanching), 2 (obvious blanching not extending the circle), to 3 (intense blanching extending over the margin of the circle). Interobserver agreement showed a reliability of r=.81. This method was previously used in different studies (Walker et al 1997; Walker et al 1998), and blanching score ratings have been validated against objective recordings with reflectance spectrophotometry

(Noon et al 1996). A sum score was computed for each subject's response to beclomethasone as the total of the six blanching scores.

4.3.4 Dexamethasone Suppression of IL-6 Production in Leukocytes

Venous blood was collected in heparinized sterile tubes (Braun, Melsungen, Germany) and diluted 10:1 with saline. The blood was then coincubated with lipopolysaccharide (LPS, E. Coli, Difco, Augsburg, Germany) and six different concentrations of Dex (Sigma, Deisenhofen, Germany) on a 24-well plate (Greiner, Nuertingen, Germany). Diluted whole blood (400 µl) was added to 50 µl of LPS and 50 µl of Dex. The final concentrations were 30 ng/ml LPS and 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-5} M Dex, respectively. After 6 h of incubation at 37°C and 5% CO_2, the plates were centrifuged for 10 min at 2000 g at 4°C. Culture supernatant was harvested and stored at -80°C until analysis.

4.3.5 Biochemical Analyses

Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendörfer et al 1992). ACTH was measured in EDTA plasma with a chemiluminescence immunoassay (Nichols institute, Bad Nauheim, Germany). Plasma Dexamethasone was assessed with an in-house RIA at the Institute of Pharmacology, University of Heidelberg. IL-6 was measured using ELISA employing the multiple antibody sandwich principle (BD Pharmingen, San Diego, CA, USA). Interassay and intraassay coefficients of variance were below 10% and 12%, respectively, for all analyses.

4.3.6 DNA Extraction and Genotyping

DNA was extracted from 10 ml peripheral venous blood following a standard NaCl salting out method according to the protocol of Miller (1988). Genotyping was performed using the allelic discrimination technique, with custom designed primers and probes (Assay by Design service, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands, primer and probe sequences available on request), using TaqMan Universal PCR master mix (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions.

4.3.7 Statistical Analyses

First, all data were log transformed to yield unskewed outcome variables. General Linear Models (GLMs) were computed to assess the repeated measures effect *time*, the between-subjects effect *genotype* as well as the interaction *time x genotype* for

salivary cortisol levels following the DST. For analysis of cytokine production, a GLM with repeated measure concentration of Dex was computed. In order to reveal possible *sex* by *genotype* interactions, *sex* was also included as predictor in the GLMs. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. One-way ANOVAs were performed to compare mean plasma ACTH and Dex levels, the area under the curve (AUC) for salivary cortisol levels after Dex administration as well as the IC_{50} for IL-6 between experimental groups. The AUC was computed with reference to zero (Wüst et al 2000) for saliva samples at 0800, 1100, 1500 and 2000h. IC_{50} values were determined by plotting a curve of the cytokine production using an exponential fit with r^2>.90. Skin Blanching scores with rising beclomethasone concentration and the sum score were compared using the Kruskal-Wallis test. Rank order correlations of the GC sensitivity markers were performed. All results shown are the mean ± SEM.

4.4 Results

4.4.1 Genotyping

Genotyping of 601 subjects for the four polymorphisms under investigation revealed five haplotypes (see Figure 4.1). Allelic frequencies observed correspond to those previously reported and all SNPs were in Hardy Weinberg Equilibrium.

The allele with the highest prevalence (45%) will be referred to as the *Most Common Allele* (MCA, allele 1). The GAGAGG to GAAAAG transition at position 22/23 (2.5%) always occurred together with the base change from A to G in exon 9beta (allele 2), which, in 13.5% of the subjects, was also observed independently from 22/23 and the other markers (allele 5). The base changes from A to G at position 363 (4%, allele 3) and the intronic change from C to G (*Bcl*I, 35%, allele 4) also occurred independently of the other investigated variants. Individual haplotype assignments for the 4 polymorphisms were determined using SNPHAP. Only haplotype assignments with a remaining uncertainty of less than five percent and haplotypes with a frequency over 2 percent were included in the analyses. Linkage disequilibrium among the 4 markers was estimated with D' and r2 using Haploview. The genotype distribution of the 206 phenotyped subjects is indicated in Table 4.1.

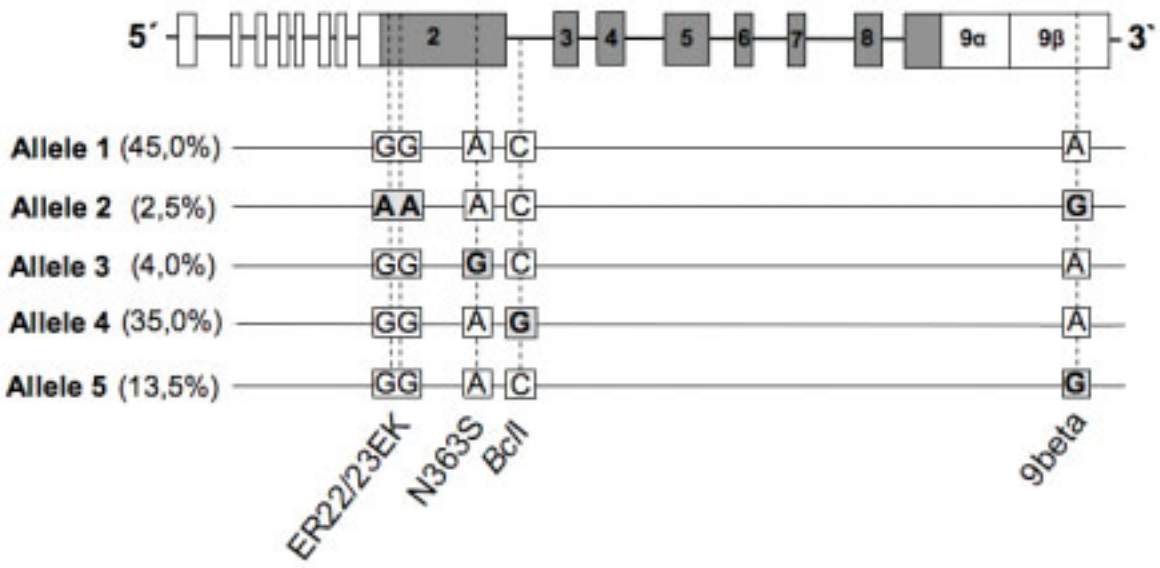

Figure 4.1: *Genomic organization of the human glucocorticoid receptor gene (NR3C1). Exons are indicated by boxes and the translated part of the gene is shown in darker shade. Lower part of the figure shows allelic frequencies of the SNPs and the observed haplotype structure. Base pair substitution are denoted by bold letters*

Genotype groups	Alleles	N	Women	Men
MCA homozygotes	Allele 1 / Allele 1	31	19	12
*Bcl*I CG	Allele 1 / Allele 4	62	35	27
*Bcl*I GG	Allele 4 / Allele 4	37	24	13
9beta AG	Allele 1 / Allele 5	31	18	13
N363S AG	Allele 3 carriage	27	14	13
ER22/23EK GA	Allele 2 carriage	18	8	10
	Total	**206**	**118**	**88**

Table 4.1: *Genotype groups, frequencies and allele combinations. Due to low prevalence of the 9beta G, N363S G and ER22/23EK A alleles, no homozygotes for the respective alleles were investigated. In the 9beta AG group, carriers of the 9beta G allele (allele 5) were only paired with the Most Common Allele (MCA, allele1). In the N363S AG and ER22/23EK group, the variant alleles were paired with allele 1 and allele 4*

4.4.2 Dexamethasone Suppression Test

Sex effect

A significant sex effect emerged for post Dex ACTH and salivary cortisol levels. Men showed higher levels of ACTH at 0800 h ($F_{1,212}$=21.6, p=.0001) as well as higher salivary cortisol levels following awakening (main effect *sex*: $F_{1,196}$=36.7, p=.0001; interaction *sex* x *time*: $F_{2.2,421.9}$=10.9, p=.0001) and in the diurnal profile (main effect *sex*: $F_{1,181}$=6.7, p=.01; interaction *sex* x *time*: $F_{1.9,340.3}$=4.14, p=.02).

Genotype and genotype by sex effects

Post Dex ACTH levels did not differ between GR genotype groups in females, while in men, 9beta AG carriers displayed the highest ACTH levels (Figure 4.2), resulting in a significant *sex* by *genotype* interaction ($F_{5,202}$=2.5, p=.03).

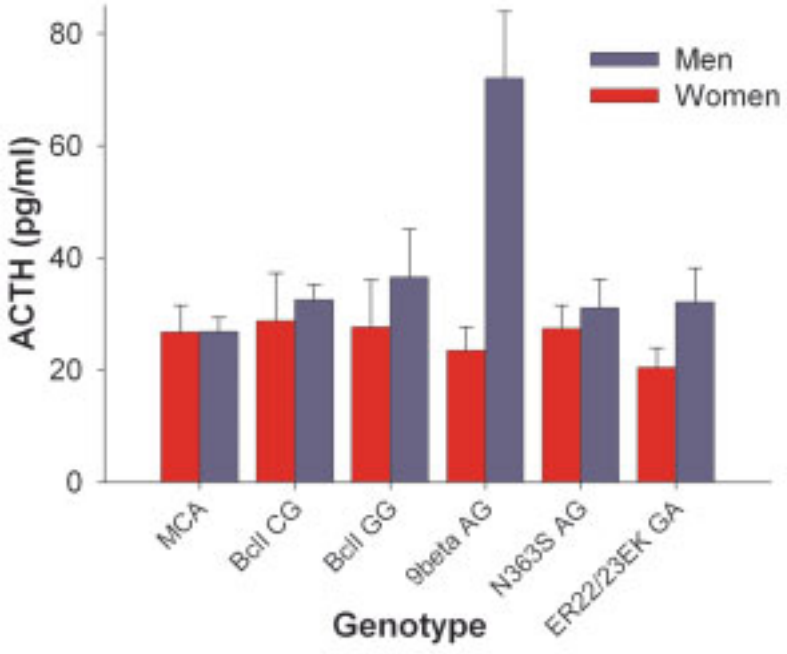

Figure 4.2: *ACTH levels at 0800 h following 0.25 mg Dexamethasone ingestion in the six genotype groups*

Figure 4.3 shows the cortisol awakening response (CAR) and salivary cortisol levels during the course of the day in men (4.3 a) and women (4.3 b). In men, the relative non-suppression of ACTH levels in the 9beta AG genotype is reflected in the post Dex cortisol awakening rise (CAR) with 9beta AG men showing the largest cortisol increase and, together with male *Bcl*I GG carriers, the highest post Dex cortisol peak levels (main effect *genotype*: $F_{5,174}$=2.3, p=.05). Interestingly, 9beta AG women were among the group showing lowest levels, again pointing towards sex specific effects of the 9beta polymorphism (interaction *sex* x *genotype*: $F_{5,174}$=.69, p=.63). In the

course of the day, cortisol levels in men were very similar for all genotype groups except for the 9beta AG genotype showing elevated levels at 1500 h and the 22/23EK carriers displaying elevated levels at all time points (Interaction *genotype* x *time*: $F_{9.4,340.3}$=1.69, p=.08). In women, the genotype groups with relatively higher levels after awakening (N363S, *Bcl*I CG and GG) also showed higher levels during the day in comparison to the other groups (MCA, 9beta AG, ER22/23EK). A tendency towards a *sex* by *genotype* interaction was observed for the diurnal profile ($F_{5,181}$=1.79, p=.12).

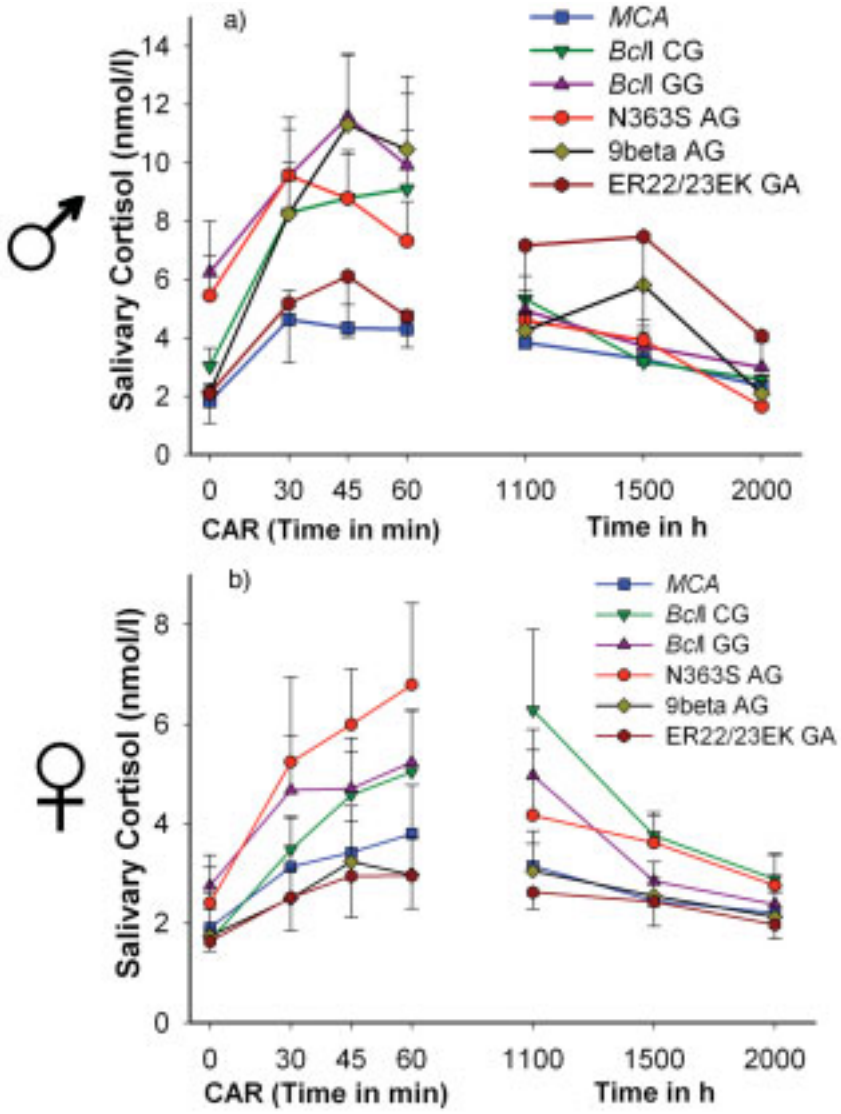

Figure 4.3: *Left parts of the figures show the Cortisol Awakening Response (CAR) for all genotype groups in* ***a)*** *men and* ***b)*** *women. Right part of the figure indicates salivary cortisol levels at three time points in the course of the day*

In Figure 4.4 total cortisol production after Dex suppression is expressed as area under the curve for the diurnal profile. Although not yielding statistical significance

(Main effect *genotype*: $F_{5,174}$=.76, p=.56; Interaction *genotype* x *sex*: $F_{5,174}$=1.47, p=.20), male 22/23EK carriers clearly secreted the largest amount of cortisol throughout the day.

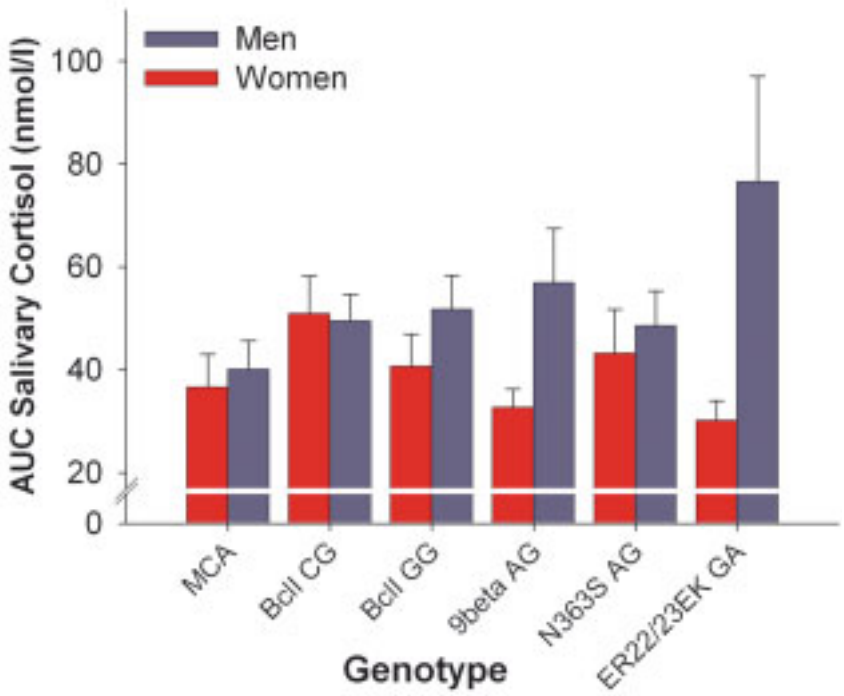

Figure 4.4: *Salivary cortisol secretion after ingestion of 0.25 mg Dex expressed as area under the curve of the diurnal profile*

4.4.3 Skin Blanching

The intensity of skin blanching increased with rising concentrations of beclomethasone. No sex differences emerged for blanching scores (all $X^2_{(1)}$<2.48 and all p>.15), thus, the mean intensity of skin blanching in relation to increasing concentrations of beclomethasone is depicted in Figure 4.5 a for the genotype groups averaged across sex. Significant differences between genotypes became evident at concentrations of 1, 10 and 20 µg/ml beclomethasone, respectively ($X^2_{(5)}$>12.19, p<.03; $X^2_{(5)}$>19.77, p<.001; $X^2_{(5)}$>12.19, p<.03. Bonferroni adjusted p value: .008). Homozygous *Bcl*I G carriers displayed the lowest intensity of skin blanching at concentrations of 1, 5, 10 and 20µg/ml of beclomethasone, reflecting a lesser degree of vasoconstriction in response to the GC. Figure 4.5 b depicts differences in the skin blanching sum score between the genotype groups ($X^2_{(5)}$=15.07, p=.01).

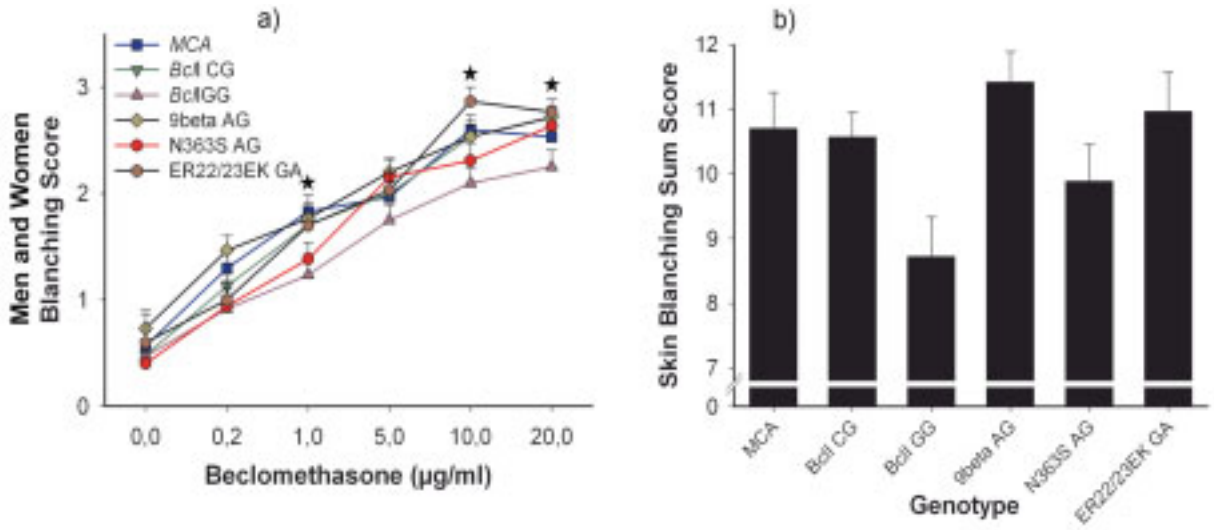

Figure 4.5: *Blanching scores in relation to beclomethasone concentration in the GR genotype groups are shown in Figure* ***a)****. In Figure* ***b)****, blanching scores are expressed as the total of scores obtained at different concentrations (*p<.03).*

4.4.4 GC sensitivity of cytokine production

LPS induced secretion of IL-6 was dose-dependently inhibited by Dex ($F_{2.2,384.6}$=454.01, p=.0001). IL-6 production in response to LPS and suppression of the secretion by Dex is shown in Figure 4.6 a and b for men and women, respectively. There was a trend for a main effects *sex* ($F_{1,177}$=2.39, p=.12) with men showing higher IL-6 levels in response to LPS stimulation (not shown in a figure). In addition, there was a trend for a main effect *genotype* ($F_{1,177}$=1.66, p=.15) and a trend for a *sex* by *genotype* interaction ($F_{5,177}$=2.15, p=.06). Both in men and women, there was large variability in IL-6 secretion between the genotype groups in response to LPS without conincubation with Dex, possibly reflecting differences in cytokine production between GR genotype groups and further pointing towards a sex specific effect of GR genotype on IL-6 secretion. In men, the 9beta AG genotype was among the groups with the lowest IL-6 secretion in response to LPS while 9beta AG women, together with the MCA genotype, showed the highest IL-6 levels.

However, no differences in the efficiency of Dex to suppress cytokine production could be revealed between men and women and between the genotype groups (interaction *genotype* by *Dex*: $F_{10.9,384.6}$=.43, p=.94; interaction *sex* by *Dex*: $F_{2.2,384.6}$=.37, p=.71).

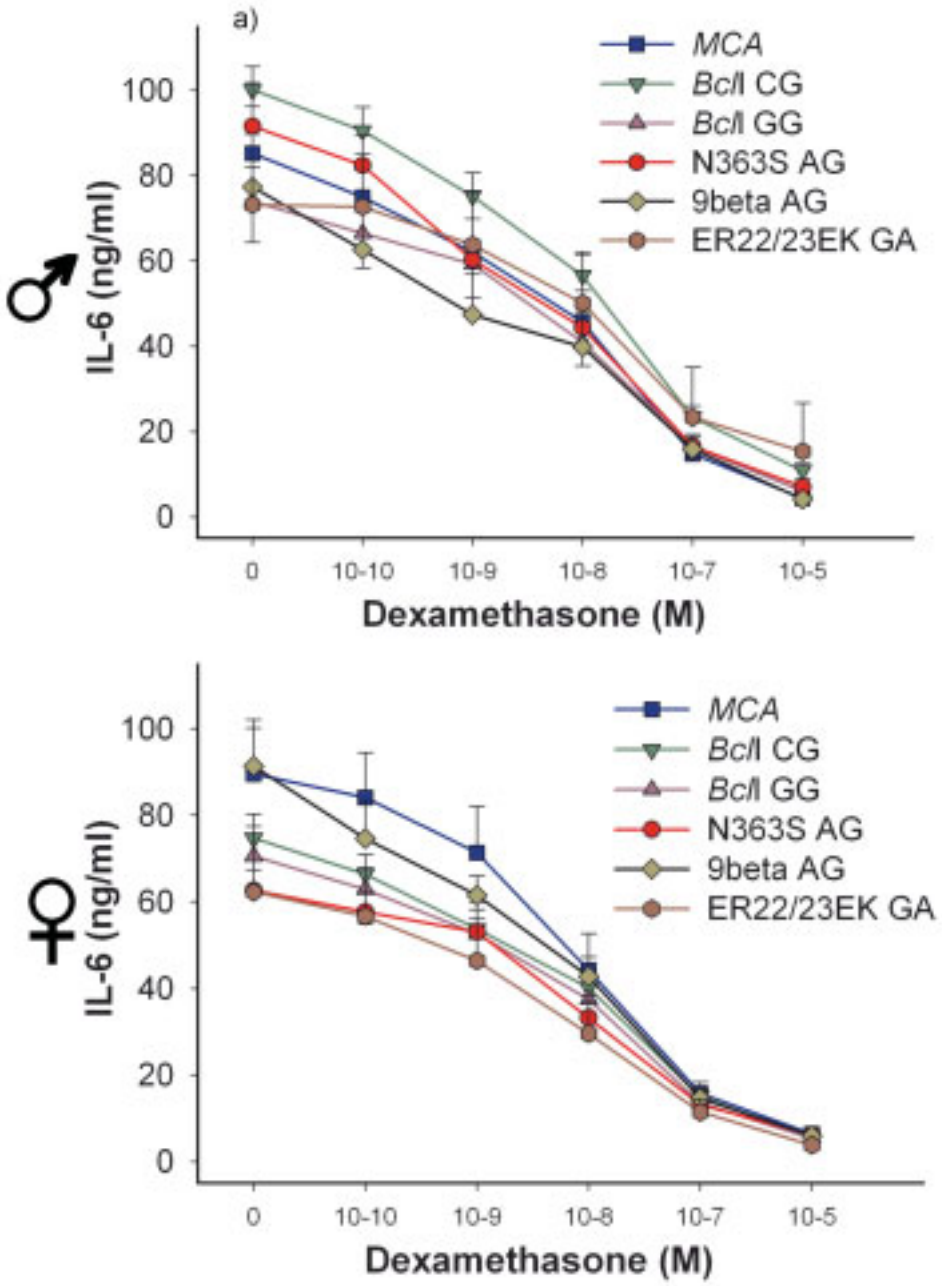

Figure 4.6: *LPS stimulated production and Dex inhibition of IL-6 in whole blood cultures in the GR genotype groups shown for* ***a)*** *men and* ***b)*** *women*

This is further reflected in IC_{50} values for IL-6, shown in Figure 4.7. The IC_{50} represents the concentration of Dex required for 50% inhibition of LPS induced cytokine production and is inversely related to GC sensitivity, i.e. a higher IC_{50} indicates a lower sensitivity and vice versa. Although the IC_{50} value in male 9beta AG carriers and in the *Bcl*I CG genotype was higher compared with the other groups, no significant effects were revealed (main effect *genotype*: $F_{5,170}$=1.35, p=.25; interaction *genotype* x *sex*: $F_{5,174}$=.69, p=.63).

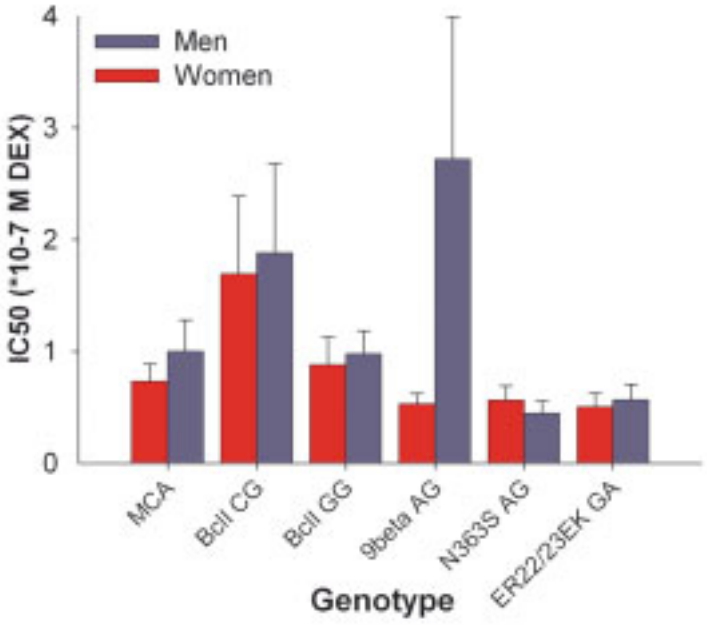

Figure 4.7*: GC sensitivity of leukocytes in the genotype groups. Lower IC_{50} values indicate a higher sensitivity and vice versa*

4.4.5 Intercorrelations

Intercorrelations between the different markers of GC sensitivity were analyzed for the overall group and separately for men and women. In addition, correlations were analyzed for each genotype group, also separately for men and women. As shown in Table 4.2 a, no significant correlations emerged for the three markers in the overall group or in men. In women, a significant negative correlation (r_s=-.21, p=.04) was found between post Dex cortisol levels expressed as AUC (Dex AUC) and skin blanching sum scores (SumSkin). Since low post Dex cortisol levels and high skin blanching scores both reflect a higher GC sensitivity, a negative correlation reflects a positive association in GC sensitivity between the two tissues. When analyzed separately for genotype groups, correlation coefficients for Dex AUC and SumSkin showed a range between r_s=.01 (MCA group) and r_s=-.40 (*Bcl*I GG group). Both the *Bcl*I CG heterozygotes (r_s=-.35, p=.06) and the *Bcl*I G homozygotes (r_s=.40, p=.16) showed larger correlations than the overall group, although only a trend for statistical significance was observed, most likely due to the limited sample size. Correlations between Dex AUC and IC_{50} IL-6 were low in all genotype groups and no statistical significance was revealed (all p >.4). Relating IC_{50} IL-6 values to the skin blanching sum score, a significant correlation was observed in female 22/23EK carriers (r_s=-.83 p=.02). Low IC_{50} values reflect a higher sensitivity; therefore, a negative correlation indicates a positive association.

In men, significant associations were revealed for the *Bcl*I GG, the N363S GA and the ER22/23EK groups. In the *Bcl*I GG group, a positive correlation was observed between Dex AUC and IC_{50} IL-6 values (r_s=.75, p=.005), i.e., high GC sensitivity on pituitary level is related to high GC sensitivity of leukocytes in this genotype. However, the observed correlation between IC_{50} IL-6 and SumSkin (r_s=.59, p=.03) reflects an inverse relationship in GC sensitivity between the two tissues, i.e., a higher sensitivity of blood vessels is related to lower sensitivity of leukocytes and vice versa. The same direction in the association between IC_{50} IL-6 and SumSkin was observed in N363S AG carriers (r_s=.60, p=.03). In the ER22/23EK GA genotype, a positive correlation between Dex AUC and IC_{50} IL-6 was observed (r_s=.94, p=.005).

Table 4.2: *Correlations between the GC sensitivity markers - IC_{50} values for Dex inhibition of IL-6 production, salivary cortisol production expressed as area under the curve following Dex ingestion (Dex AUC), and total blanching score after topical beclomethasone application (SumSkin) – are shown for the overall group and seperately for men and women in Table 4.2 a). Correlations are shown seperately for genotype groups for women in Table 4.2 b) and for men in Table 4.2 c)*

Table 4.2 a): Correlations of GC Sensitivity Markers

		Dex AUC	IC_{50} IL-6
Overall group	**SumSkin**		
	r_s	-.11	.06
	p	.18	.43
	IC_{50} IL-6		
	r_s	.12	
	p	.11	
		Dex AUC	**IC_{50} IL-6**
Women	**SumSkin**		
	r_s	-.21*	-.09
	p	.04	.38
	IC50 IL-6		
	r_s	.13	
	p	.20	
		Dex AUC	**IC_{50} IL-6**
Men	**SumSkin**		
	r_s	-.09	.2
	p	.48	.09
	IC_{50} IL-6		
	r_s	.05	
	p	.68	

Table 4.2 b): Correlations of GC Sensitivity Markers in Women

Genotype		Dex AUC	IC_{50} IL-6
MCA	**SumSkin**		
	r_s	.01	.28
	p	.97	.33
	IC_{50} IL-6		
	r_s	.25	
	p	.42	
		Dex AUC	**IC_{50} IL-6**
***Bcl*I CG**	**SumSkin**		
	r_s	-.35	-.22
	p	.06	.23
	IC50 IL-6		
	r_s	-.05	
	p	.78	
		Dex AUC	**IC_{50} IL-6**
***Bcl*I GG**	**SumSkin**		
	r_s	-.4	-.21
	p	.16	.41
	IC_{50} IL-6		
	r_s	.03	
	p	.93	
		Dex AUC	**IC_{50} IL-6**
9beta AG	**SumSkin**		
	r_s	.06	-.15
	p	.84	.59
	IC_{50} IL-6		
	r_s	.17	
	p	.57	
		Dex AUC	**IC_{50} IL-6**
N363S AG	**SumSkin**		
	r_s	-.26	.22
	p	.45	.5
	IC_{50} IL-6		
	r_s	.10	
	p	.77	
		Dex AUC	**IC_{50} IL-6**
ER22/23EK GA	**SumSkin**		
	r_s	-.2	-.83*
	p	.67	.02
	IC_{50} IL-6		
	r_s	.00	
	p	1	

Table 4.2 c): Correlations of GC Sensitivity Markers in Men

Genotype		Dex AUC	IC_{50} IL-6
MCA	**SumSkin**		
	r_s	-.14	.22
	p	.73	.61
	IC_{50} IL-6		
	r_s	.36	
	p	.39	
		Dex AUC	**IC_{50} IL-6**
***Bcl*I CG**	**SumSkin**		
	r_s	-.1	.14
	p	.65	.53
	IC50 IL-6		
	r_s	-.36	
	p	.1	
		Dex AUC	**IC_{50} IL-6**
***Bcl*I GG**	**SumSkin**		
	r_s	.34	.59*
	p	.28	.03
	IC_{50} IL-6		
	r_s	.75**	
	p	.005	
		Dex AUC	**IC_{50} IL-6**
9beta AG	**SumSkin**		
	r_s	-.24	-.5
	p	.47	.12
	IC_{50} IL-6		
	r_s	.05	
	p	.86	
		Dex AUC	**IC_{50} IL-6**
N363S AG	**SumSkin**		
	r_s	-.38	.60*
	p	.25	.03
	IC_{50} IL-6		
	r_s	-.29	
	p	.39	
		Dex AUC	**IC_{50} IL-6**
ER22/23EK GA	**SumSkin**		
	r_s	.80	.67
	p	.20	.22
	IC_{50} IL-6		
	r_s	.94**	
	p	.005	

4.5 Discussion

There is wide variety in GC sensitivity between individuals, and even within the same individual, GC responsiveness varies between tissues. In the present study, the effect of four common GR gene polymorphisms on GC sensitivity was studied in healthy individuals. GR gene variants were associated with responses to exogenous

GCs in two target tissues. Following a low dose (0.25 mg) Dex suppression test, male 9beta AG carriers displayed a relative non-suppression of ACTH while no differences between genotypes were observed in women. 9beta AG men also showed the highest increase and the highest peak levels in post Dex salivary cortisol levels in the morning. In the course of the day, male ER22/23EK AG carriers displayed an escape from Dex suppression, while female carriers of the same genotype showed suppressed levels throughout the day, further indicating sex specific effects of GR gene variants in the Dex suppression test. In the skin blanching test, the *Bcl*I GG genotype group in both men and women showed the least degree of skin blanching, reflecting a lower GC sensitivity of subdermal blood vessels in this genotype. No association between GR genotype and GC sensitivity of peripheral leukocytes was observed. While there was a trend for a sex effect and a sex by genotype effect for IL-6 production in response to LPS, no sex or sex by genotype effect became evident for Dex mediated cytokine suppression. Intercorrelations between the three GC sensitivity markers were highly variable between men and women and the genotype groups. Significant correlations were observed for *Bcl*I GG and N363S AG genotype groups in men and for ER22/23EK GA carriers in men and women, while in the remaining genotype groups, no significant correlations in GC sensitivity was observed between the three target tissues.

The N363S, the *Bcl*I and the ER22/23EK polymorphisms have previously been associated with sensitivity to exogenous GC administration. The 363S allele and the *Bcl*I G allele were related to increased responses to Dex (Huizenga et al 1998a; Van Rossum et al 2003) whereas the 22/23EK allele was associated with decreased responses to Dex (van Rossum et al 2002). In the present study, these findings could not be confirmed for the N363S and the *Bcl*I genotype and only in part for the ER22/23EK genotype. In women, salivary cortisol levels following awakening and throughout the day were higher in the N363S and the *Bcl*I genotype compared with the remaining groups. In men, the N363S AG group and carriers of the *Bcl*I G allele showed higher levels in comparison to the MCA and 22/23EK group in the morning, while afternoon levels were low. The ER22/23EK AG genotype in men had, together with the MCA groups, the lowest salivary cortisol levels in the morning, which is in contrast to the reduced sensitivity as observed in previous studies. However, male 22/23EK carriers showed elevated cortisol levels in the course of the day, suggesting a relative escape from Dex suppression, which is in accordance to the suggested relative GC insensitivity in this genotype. Interestingly, no such escape could be

observed in the same genotype in women. No significant differences in ACTH levels could be observed between the N363S, *Bcl*I and the ER22/23EK genotype. The observed discrepancies between results from the present and previous studies may be due to differences in study populations, since the previous studies were carried out in an elderly population (>55 years old) and did not report results separately for men and women. In addition, the previously detected association between the ER22/23EK polymorphism and GC sensitivity was observed after administration of 1 mg Dexamethasone and not after 0.25 mg as used in the present study. Furthermore, in previous studies serum total cortisol was measured, whereas the present study assessed ACTH and salivary cortisol levels. The strongest effects for ACTH and salivary cortisol responses following Dex suppression were observed for 9beta AG carriers in men. The 9beta variant has been shown to increase GRbeta mRNA stability, possibly leading to higher expression of GRbeta protein (Derijk et al 2001), which serves as a dominant negative inhibitor of GRalpha (Charmandari et al 2005; Oakley et al 1996; Schaaf and Cidlowski 2002; Yudt et al 2003). Furthermore, a lower transrepressing activity has been reported for this variant (van den Akker et al 2006b), and a recent study observed a lower risk of persistent *Staphylococcus Aureus* nasal carriage in 9beta GG carriers, possibly resulting from reduced GC-induced immune suppression (van den Akker et al 2006a). Our data suggest a relative GC insensitivity on pituitary level in male 9beta AG carriers as indicated by enhanced ACTH and cortisol levels following the DST. These observations are in accordance with the functional analyses and observation from association studies. Although very low levels of GRbeta mRNA and protein have been observed in hippocampus (DeRijk et al 2003) and other tissues (Pujols et al 2001), it has been demonstrated that under certain conditions levels of GRbeta mRNA and protein can be upregulated. The factor necessary for alternative splicing of the GR pre-mRNA was identified as SRp30c (serine-arginine-rich protein 30c; Xu et al 2003). Different cell types show varying levels of GRbeta, which might be due to different levels of SRp30c expression. Treatment of neutrophils with IL-8 leads to enhanced expression of both SRp30c and GRbeta, furthermore, inflammatory processes (Webster et al 2001) increase the abundance of GRbeta mRNA and protein.

GC sensitivity of subdermal blood vessels to the synthetic GC beclomethasone was lower in *Bcl*I GG genotype in both sexes compared to the other groups. These results confirm previous findings that had observed lowest blanching scores reflecting the lowest degree of vasoconstriction in the *Bcl*I GG genotype (Panarelli et al 1998). No

data on putative functional mechanisms are available for the intronic *Bcl*I polymorphism.

Two previous studies have associated GR gene polymorphisms with GC sensitivity of leukocytes. Panarelli et al. (1998), using Dex sensitive lysozyme release from leukocytes as an indicator of GC sensitivity, observed a tendency towards higher GC sensitivity in the *Bcl*I GG genotype (n=7) compared to CG heterozygotes and CC homozygotes. Huizinga et al. (1998a) compared the sensitivity of leucocytes to Dex between carriers and non-carriers of the N363S allele and reported a trend for increased sensitivity in nine individuals carrying the 363S allele. Both studies did not reveal statistically significant differences, which might be due to the limited sample sizes. The present study, with slightly higher sample sizes, could not confirm the observed trends reported before.

Our results suggest that different GR gene variants can have tissue specific effects on GC sensitivity, i.e., polymorphisms of the GR can alter GC sensitivity in one target tissue and have no effect in other GC responsive cells. In addition, correlations in GC sensitivity between tissues are also GR genotype specific, since significant correlations emerge for three GR genotypes in a sex specific way. It has been demonstrated before that GC sensitivity is highly variable between tissues. No correlation in GC sensitivity between tissues has been observed in healthy individuals (Ebrecht et al 2000; Vasiliadi et al 2002) and in clinical studies (Corrigan et al 1991; Corrigan et al 1996; Sher et al 1994). When analyzed for the entire group, these results are confirmed by the present study. However, when analyzed separately for genotype groups, both positive and negative associations between GC sensitivity markers are revealed. For the *Bcl*I GG genotype in men, there was a positive association between IC_{50} IL-6 values and Dex suppressed cortisol levels, and at the same time, an inverse association between IC_{50} IL-6 values and skin blanching sum score was detected. In male N363S AG carriers, IC_{50} IL-6 values and the skin blanching sum score were also inversely related, i.e., high GC sensitivity of leukocytes was associated with low sensitivity of subdermal blood and vice versa. In male ER22/23EK GA carriers, there was a positive association between IC_{50} IL-6 values and Dex suppressed cortisol levels, while in women, a positive association became evident between skin blanching scores and IC_{50} IL-6 values.

GCs have a multitude of effects in different physiological systems but exert their effects mainly through one receptor, the GR. The molecular basis for the observed variability in GC responsiveness can be partially attributed to existence of isoforms of

the GR resulting from alternative splicing, alternative translation initiation and posttranslational modifications (Lu and Cidlowski 2006). Different cellular environments are likely to result in differential expression of GR isoforms, all possessing unique transcriptional regulatory profiles. Tissue specific GR isoform compositions could determine the cell-specific response to glucocorticoids thus accounting for the diverse and specific effects of GCs (Lu and Cidlowski 2006). The molecular mechanisms underlying GR isoform generation have been identified (Lu and Cidlowski 2005), however, how tissues direct this expression to achieve their unique cell specific isoform composition is largely unknown. It has been shown that presence of GR gene variants can impact on the regulation of isoform expression and thus alter the cellular composition and proportion of these isoforms. This has been demonstrated for the ER22/23EK variant, which leads to an enhanced expression of the transcriptionally less active isoform GR-B (Russcher et al 2005b) and for the 9beta variant, whose stabilizing effect of 9beta mRNA leads to an enhanced expression of the GRbeta isoform (Derijk et al 2001). Thus, GR gene variants might play an important role in the fine-tuning of the GC response via effects on GR isoform expression and these effects might be differentially pronounced in different GC responsive cells.

A distinct sex and sex specific genotype effect was observed in post Dex ACTH and salivary cortisol levels, while only a trend for a sex and sex by genotype effect was detected in cytokine production following LPS stimulation and no sex or sex by genotype effect could be observed for GC induced vasoconstriction. It has to be noted that all female participants were using ethinyl-estradiol containing oral contraceptives (OCs). It is well known that ethinyl-estradiol leads to increases in corticosteroid binding globulin (CBG) synthesis (Wiegratz et al 2003), which in turn influences the availability of free cortisol (Kirschbaum et al 1999; Kirschbaum et al 1995). However, OC intake resulting in high levels of CBG are most likely not responsible for our observed genotype by sex interactions, since all women were taking OCs and we did not observe significantly different CBG levels between the genotype groups ($p=.30$). Furthermore, whereas differences in salivary cortisol levels between men and women following Dex suppression can in part be explained by elevated CBG levels, ACTH levels were similar in men and women except for the 9beta AG genotype, making it unlikely that the observed sex by genotype effect is due to OC intake.

In summary, we investigated the association of common GR gene polymorphisms with GC sensitivity of three important GC target tissues, namely blood vessels, the immune system and the pituitary. We observed associations of GR polymorphisms variants with markers of GC sensitivity in a sex and tissue specific way. GC responsiveness is highly variable between individuals and also between tissues within the same individual. Our results suggest that genetic variation of the GR is one important factor in the explanation of these differences.

4.6 References

Baxter JD, Rousseau GG (1979): Glucocorticoid hormone action: an overview. *Monogr Endocrinol* 12:1-24.

Charmandari E, Chrousos GP, Ichijo T, Bhattacharyya N, Vottero A, Souvatzoglou E, Kino T (2005): The human glucocorticoid receptor (hGR) beta isoform suppresses the transcriptional activity of hGRalpha by interfering with formation of active coactivator complexes. *Mol Endocrinol* 19:52-64.

Charmandari E, Kino T, Chrousos GP (2004): Familial/sporadic glucocorticoid resistance: clinical phenotype and molecular mechanisms. *Ann N Y Acad Sci* 1024:168-181.

Corrigan CJ, Brown PH, Barnes NC, Szefler SJ, Tsai JJ, Frew AJ, Kay AB (1991): Glucocorticoid resistance in chronic asthma. Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids in vitro. *Am Rev Respir Dis* 144:1016-1025.

Corrigan CJ, Bungre JK, Assoufi B, Cooper AE, Seddon H, Kay AB (1996): Glucocorticoid resistant asthma: T-lymphocyte steroid metabolism and sensitivity to glucocorticoids and immunosuppressive agents. *Eur Respir J* 9:2077-2086.

DeRijk R, Sternberg EM (1997): Corticosteroid resistance and disease. *Ann Med* 29:79-82.

DeRijk RH, Schaaf M, Stam FJ, de Jong IE, Swaab DF, Ravid R, et al (2003): Very low levels of the glucocorticoid receptor beta isoform in the human hippocampus as shown by Taqman RT-PCR and immunocytochemistry. *Brain Res Mol Brain Res* 116:17-26.

Derijk RH, Schaaf MJ, Turner G, Datson NA, Vreugdenhil E, Cidlowski J, et al (2001): A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol* 28:2383-2388.

Dressendorfer RA, Kirschbaum C, Rohde W, Stahl F, Strasburger CJ (1992): Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. *J Steroid Biochem Mol Biol* 43:683-692.

Ebrecht M, Buske-Kirschbaum A, Hellhammer D, Kern S, Rohleder N, Walker B, Kirschbaum C (2000): Tissue specificity of glucocorticoid sensitivity in healthy adults. *J Clin Endocrinol Metab* 85:3733-3739.

Fleury I, Beaulieu P, Primeau M, Labuda D, Sinnett D, Krajinovic M (2003): Characterization of the *Bcl*I polymorphism in the glucocorticoid receptor gene. *Clin Chem* 49:1528-1531.

Hamid QA, Wenzel SE, Hauk PJ, Tsicopoulos A, Wallaert B, Lafitte JJ, et al (1999): Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 159:1600-1604.

Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, et al (1998a): A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *J Clin Endocrinol Metab* 83:144-151.

Huizenga NA, Koper JW, de Lange P, Pols HA, Stolk RP, Grobbee DE, et al (1998b): Interperson variability but intraperson stability of baseline plasma cortisol concentrations, and its relation to feedback sensitivity of the hypothalamo-pituitary-adrenal axis to a low dose of Dexamethasone in elderly individuals. *J Clin Endocrinol Metab* 83:47-54.

Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH (1999): Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med* 61:154-162.

Kirschbaum C, Pirke KM, Hellhammer DH (1995): Preliminary evidence for reduced cortisol responsivity to psychological stress in women using oral contraceptive medication. *Psychoneuroendocrinology* 20:509-514.

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, et al (1997): Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. *Hum Genet* 99:663-668.

Lamberts SW, Huizenga AT, de Lange P, de Jong FH, Koper JW (1996): Clinical aspects of glucocorticoid sensitivity. *Steroids* 61:157-160.

Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, et al (1997): Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med* 186:1567-1574.

Lu NZ, Cidlowski JA (2005): Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell* 18:331-342.

Lu NZ, Cidlowski JA (2006): Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol* 16:301-307.

Miller SA, Dykes DD, Polesky HF (1988): A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.

Munck A, Guyre PM, Holbrook NJ (1984): Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev* 5:25-44.

Noon JP, Evans CE, Haynes WG, Webb DJ, Walker BR (1996): A comparison of techniques to assess skin blanching following the topical application of glucocorticoids. *British Journal of Dermatology* 134:837-842.

Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA (1999): The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem* 274:27857-27866.

Oakley RH, Sar M, Cidlowski JA (1996): The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *J Biol Chem* 271:9550-9559.

Panarelli M, Holloway CD, Fraser R, Connell JM, Ingram MC, Anderson NH, Kenyon CJ (1998): Glucocorticoid receptor polymorphism, skin vasoconstriction, and other metabolic intermediate phenotypes in normal human subjects. *J Clin Endocrinol Metab* 83:1846-1852.

Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH (2003): Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology* 28:916-931.

Pujols L, Mullol J, Perez M, Roca-Ferrer J, Juan M, Xaubet A, et al (2001): Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. *Am J Respir Cell Mol Biol* 24:49-57.

Russcher H, Smit P, van den Akker EL, van Rossum EF, Brinkmann AO, de Jong FH, et al (2005a): Two Polymorphisms in the Glucocorticoid Receptor Gene Directly Affect Glucocorticoid-Regulated Gene Expression. *J Clin Endocrinol Metab*.

Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW (2005b): Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. *Mol Endocrinol* 19:1687-1696.

Schaaf MJ, Cidlowski JA (2002): Molecular mechanisms of glucocorticoid action and resistance. *J Steroid Biochem Mol Biol* 83:37-48.

Sher ER, Leung DY, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ (1994): Steroid-resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. *J Clin Invest* 93:33-39.

Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ, Lee TH (2000): Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol* 105:943-950.

Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE, Donn R (2004): Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. *J Clin Endocrinol Metab* 89:892-897.

van den Akker EL, Nouwen JL, Melles DC, Rossum EF, Koper JW, Uitterlinden AG, et al (2006a): Staphylococcus aureus Nasal Carriage Is Associated with Glucocorticoid Receptor Gene Polymorphisms. *J Infect Dis* 194:814-818.

van den Akker EL, Russcher H, van Rossum EF, Brinkmann AO, de Jong FH, Hokken A, et al (2006b): Glucocorticoid receptor polymorphism affects transrepression but not transactivation. *J Clin Endocrinol Metab* 91:2800-2803.

van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, et al (2002): A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 51:3128-3134.

Van Rossum EF, Koper JW, Van Den Beld AW, Uitterlinden AG, Arp P, Ester W, et al (2003): Identification of the *Bcl*I polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. *Clin Endocrinol (Oxf)* 59:585-592.

Vasiliadi D, Gratsias Y, Tsagarakis S, Botoula E, Trivizas P, Thalassinos N, Moutsatsou P (2002): Glucocortidoid sensitivity assessed in peripheral blood cells do not correlate with the feedback sensitivity of the hypothalamo-pituitary adrenal AXIS. *Hormones (Athens)* 1:233-238.

Walker BR, Best R, Noon JP, Watt GC, Webb DJ (1997): Seasonal variation in glucocorticoid activity in healthy men. *J Clin Endocrinol Metab* 82:4015-4019.

Walker BR, Phillips DI, Noon JP, Panarelli M, Andrew R, Edwards HV, et al (1998): Increased glucocorticoid activity in men with cardiovascular risk factors. *Hypertension* 31:891-895.

Webster JC, Oakley RH, Jewell CM, Cidlowski JA (2001): Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. *Proc Natl Acad Sci U S A* 98:6865-6870.

Wiegratz I, Kutschera E, Lee JH, Moore C, Mellinger U, Winkler UH, Kuhl H (2003): Effect of four different oral contraceptives on various sex hormones and serum-binding globulins. *Contraception* 67:25-32.

Wüst S, Wolf J, Hellhammer DH, Federenko I, Schommer N, Kirschbaum C (2000): The cortisol awakening response – normal values and confounds. *Noise and Health* 7: 79-88

Wüst S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH (2004): Common Polymorphisms in the Glucocorticoid Receptor Gene Are Associated with Adrenocortical Responses to Psychosocial Stress. *J Clin Endocrinol Metab* 89:565-573.

Xu Q, Leung DY, Kisich KO (2003): Serine-arginine-rich protein p30 directs alternative splicing of glucocorticoid receptor pre-mRNA to glucocorticoid receptor beta in neutrophils. *J Biol Chem* 278:27112-27118.

Yudt MR, Jewell CM, Bienstock RJ, Cidlowski JA (2003): Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* 23:4319-4330.

CHAPTER 5

Sex Specific Associations between GR Polymorphism A3669G in Exon 9beta and Working Memory Performance

5.1 Summary

Background: Cortisol has a modulatory influence on cognitive functions in humans. Both impairing and enhancing effects of cortisol administration have been shown for hippocampus dependant declarative memory and impairing effects have been shown for prefrontal dependant working memory function.

Methods: Given the high density of glucocorticoid receptors in the prefrontal cortex, we investigated whether common variants of the glucocorticoid receptor gene (ER22/23EK, N363S, *Bcl*I, 9beta) modulate the influence of cortisol administration on working memory. Working memory performance was investigated in 162 subjects following administration of 10mg hydrocortisone and following placebo administration using an item-recognition memory task.

Results: No impairing effect of hydrocortisone treatment became evident. However, a general and sex specific effect of the 9beta AG variant on working memory performance was observed. While 9beta AG women displayed faster reaction times than male and female carriers of the other polymorphisms, 9beta AG men were relatively slower (sex by genotype interaction p=.02) compared to the other genotypes.

Conclusions: Heritability estimates for memory are roughly 50%, indicating that common genetic polymorphisms have an important impact on cognitive performance. Our results indicate that variants of the GR gene might explain some of the variance attributable to genetic factors.

5.2 Introduction

Cortisol secreted by the adrenal cortex impacts on multiple target tissues and plays an important role in the regulation of numerous processes throughout the human body. It readily enters the brain and coordinates, together with other components of the stress system, the organism´s response to challenge. Cortisol also influences affective and cognitive functions and controls the excitability of neuronal networks that underlie learning and memory processes (de Kloet et al 1999). There is abundant evidence from animal as well as from human studies that glucocorticoids (GC) can have modulatory effects on learning and memory. Studies investigating chronic exposure to high GC levels predominantly report impairing effects on memory (Belanoff et al 2001; Gold et al 2002; Lupien et al 1994; Lupien et al 1995; McEwen and Sapolsky 1995; Wolf 2003). Investigations of the acute effects of GCs, either induced by acute stress or GC treatment, report both enhancing and impairing effects of GCs on memory performance (de Kloet et al 1999; Kirschbaum et al 1996; Lupien and McEwen 1997; Lupien et al 2002; Roozendaal 2002; Wolf 2003). The majority of studies in humans assessing the impact of GCs on memory function have focused on hippocampal dependent (Oitzl et al 2001; Roozendaal 2002) declarative memory performance (Reviewed by Het et al 2005 in a recent meta analysis).

Another important target for GC action is the prefrontal cortex, indicated by high density of corticosteroid receptors, the mineralocorticoid and the glucocorticoid receptor, in both the rat (McEwen et al 1986) and human (Sarrieau et al 1988). Imaging studies have attributed various cognitive processes to the prefrontal cortex, including working memory performance (Diamond 1988; Dolan and Fletcher 1997; Fuster 1989; Smith et al 1998). Furthermore, studies in humans (Owen et al 1990; Petrides and Milner 1982) have shown that lesions of the dorsolateral prefrontal cortex lead to impairments in working memory (WM). According to Baddeley´s model (1986), WM is defined as the cognitive mechanism that underlies temporary storage and manipulation of limited amounts of information. In a study by Lupien et al. (1999), the acute effects of hydrocortisone administration on WM performance were assessed using an item-recognition task that has been reported to significantly activate prefrontal cortex in neuroimaging studies. It could be shown that the highest dose of hydrocortisone had lead to significant impairments in WM function without exerting any effect on declarative memory or arousal-vigilance. The authors

concluded from these observations that prefrontal dependent WM is more sensitive to GC administration than hippocampal dependent declarative memory.

Given the presence of glucocorticoid receptors (GR) in the human prefrontal cortex and the evidence of acute WM impairments after corticosteroid administration, the impairment of working memory performance through cortisol could be regarded as an indicator of prefrontal GC sensitivity. Among other factors, genetic variants of the GR are known to significantly influence sensitivity to GC (see van Rossum and Lamberts 2004 for review) in various tissues. The N363S (rs6195) and the *Bcl*I (no rs number assigned) variant have been associated with an increased sensitivity to exogenous GC (Huizenga et al 1998; Koper et al 1997; Russcher et al 2005) whereas the E22/23EK (rs6189&6190) and the 9beta (rs6198) polymorphism have been associated with relative GC resistance (Derijk et al 2001; Russcher et al 2005; van Rossum et al 2002). Of the known GR polymorphisms, only one has so far been studied with respect to cognitive function. The ER22/23EK variant was studied in a large study in the elderly assessing hippocampal volume, dementia and white matter lesions. While hippocampal volume was not affected, ER22/23EK carriers had a lower risk of dementia, fewer white matter lesions in the brain and better performance on psychomotor speed tests (Van Rossum and Lamberts 2004). Given a decreased sensitivity to GC of this variant, ER22/23EK carriers might be protected from detrimental effects of chronic GC exposure.

However, the effect of acute GC exposure on memory performance has not been tested in different GR genotype groups. Therefore, we studied the effects of 10mg hydrocortisone (cortisol) on working memory performance using a double-blind, placebo-controlled, within-subject design. We investigated four common GR gene variants with known functionality or previously reported associations and sufficient prevalence in the population (ER22/23EK, N363S, *Bcl*I, 9beta), in order to assess if GR gene polymorphisms modulate the effect of exogenous cortisol administration on working memory performance. Furthermore, we hypothesized a general effect of genotype on working memory performance independent of treatment, since GC induced signaling can generally differ between GR genotype groups due to differences in GC sensitivity.

5.3 Methods

5.3.1 Subjects

Subjets were recruited from a Trier-based community sample and from students of the University of Trier. An initial sample of 601 healthy subjects was recruited and genotyped for the GR variants under investigation. For working memory tests, 162 subjects (89 women and 73 men) were selected (mean age 25.1 with SEM of ± 3.9 yrs, BMI 24.2 ± 4.1). Participants were non-smokers and of central European descent. All females were using ethynil-estradiol containing oral contraceptives (OC) and all males were medication free. Prior to the first experimental session, the absence of acute or chronic diseases was confirmed in a medical exam. The study protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants.

5.3.2 Working Memory Task

Participants reported to the laboratory two times and test sessions were separated by a one-week interval. Subjects were administered 10mg hydrocortisone or placebo 1h prior to testing at 1400h to allow uptake of hydrocortisone. The order of administration was counter-balanced and randomized in a double-blind fashion. Subjects remained at the research facilities the hour before testing at 1500h and no further tests were conducted during this time period.

Working memory performance was assessed using an item-recognition task (Sternberg 1966) controlled by a computer that presented stimuli and recorded errors and reaction times. Experimental procedures were identical across test days. The task included a series of discrete trials. Each condition consisted of the presentation of three to four uppercase letters, followed by a recognition display of two to four uppercase letters, to which participants responded *yes* (present-target trials) or *no* (absent-target trials), by pressing one of two buttons on a computer keyboard, indicating whether or not one of the targets was identical to one of the stimuli in the recognition display. There was only one possible target present on the display during present-target trials. Each condition comprised 20 trials and the number of comparisons determined processing load. The processing capacity load was manipulated by varying the number of targets to be held in memory for later item recognition or by varying the number of stimuli presented in the recognition display, or by varying both. Three to four targets to be held in memory with two to four stimuli

in the recognition display lead to a range in processing load of 6 to 16 comparisons. For all conditions, the stimuli were uppercase letters and there were 20 trials per each of the five conditions, yielding a total of 100 trials. Order of processing load was randomized across participants.

5.3.3 DNA Extraction and Genotyping

DNA was extracted from 10ml peripheral venous blood following a standard NaCl salting out method according to the protocol of Miller (1988). Genotyping was performed using the allelic discrimination technique, with custom designed primers and probes (Assay by Design service, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands, primer and probe sequences available on request), using TaqMan Universal PCR master mix (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions.

5.3.4 Cortisol Measures

Salivary cortisol levels were assessed using a Salivette sampling device (Sarstedt, Nümbrecht, Germany). Saliva samples were obtained before the administration of cortisol or placebo at 1400h and immediately before testing at 1500h. Saliva samples were frozen at -20°C until analysis. Free cortisol measured in saliva was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendorfer et al 1992). Interassay and intraassay coefficients of variance were 4 – 6.7% and 7.1 - 9% respectively.

5.3.5 Statistical Analyses

For the analyses of cortisol measures, a General Linear Model (GLM) was computed with repeated measures *time of day* (1400h vs. 1500h) and *treatment* (hydrocortisone vs. placebo) and between-subject factors *genotype* and *sequence of treatment* (hydrocortisone administration on test day 1 vs. 2).

In order to analyze working memory performance, General Linear Models (GLMs) were computed with repeated measures *working load* (6 vs. 8 vs. 9 vs. 12 vs. 16). For the analysis of sequence effects, *test day* was entered as repeated measure and *sequence of treatment* was entered as an additional predictor to analyze *treatment* by *sequence of treatment* interactions. For the analysis of treatment effects, *treatment* was entered as repeated measure and *sequence of treatment* was replaced by *test day* as predictor to control for sequence effects. For the analysis of

genotype effects, the between-subjects factor *genotype* was entered. In order to reveal genotype by sex interactions, *sex* was also entered as additional predictor. Correlational analyses were conducted on the relationship between cortisol levels and reaction times for the different treatment conditions separately for test days 1 and 2. Greenhouse-Geisser corrections were applied where appropriate and only adjusted results are reported. All results shown are the mean ± SEM. Detection errors were all below 5% and were not subjected to further analyses.

5.4 Results

5.4.1 Genotypes

Genotyping of the initial 601 subjects for four polymorphic markers revealed five haplotypes (see Figure 5.1).

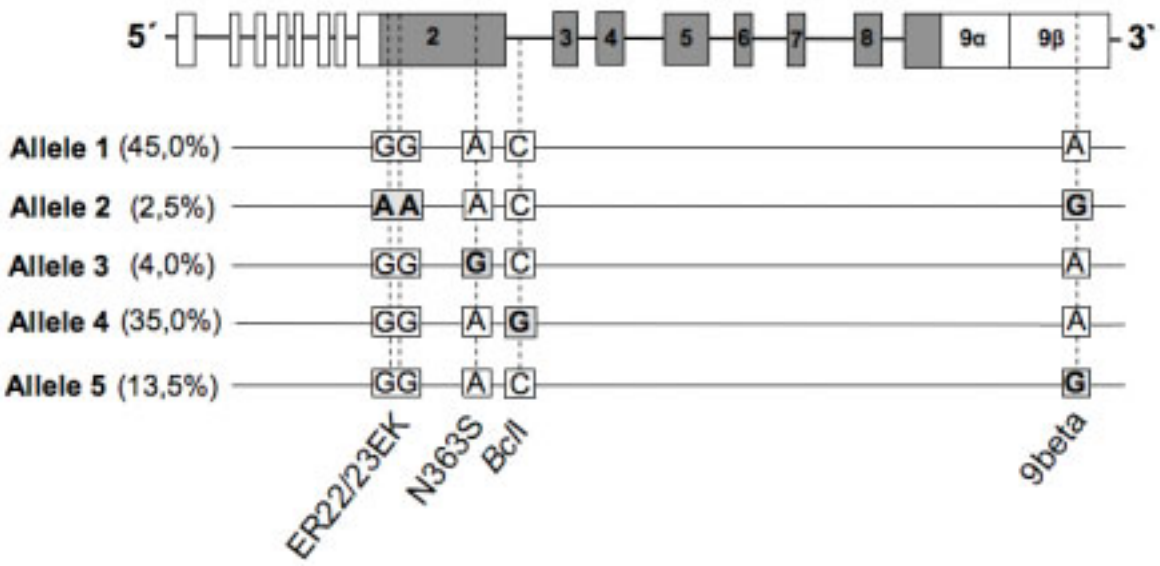

Figure 5.1: *The allele with the highest prevalence (45%) will be referred to as the Most Common Allele (MCA). The GAGAGG to GAAAAG transition at position 22/23 (2.5%) always occurred together with the base change from A to G in exon 9beta, which, in 13.5% of the subjects, was also observed independently from 22/23 and the other markers. The base changes from A to G at position 363 (4%) and the intronic change from C to G (BclI, 35%) also occurred independently of the other investigated variants*

Allelic frequencies observed correspond to those previously reported and all SNPs were in Hardy Weinberg Equilibrium. The genotype distribution of the 162 investigated subjects is indicated in Table 5.1.

Genotype groups	Alleles	N	Women	Men
MCA homozygotes	Allele 1 / Allele 1	18	12	6
*Bcl*I CG	Allele 1 / Allele 4	52	27	25
*Bcl*I GG	Allele 4 / Allele 4	30	20	10
9beta AG	Allele 1 / Allele 5	22	11	11
N363S AG	Allele 3 carriage	23	11	12
ER22/23EK GA	Allele 2 carriage	17	8	9
Total		**162**	**89**	**73**

Table 5.1: *Genotype groups, frequencies and allele combinations. Due to low prevalence of the 9beta G, N363S G and ER22/23EK A alleles, no homozygotes for the respective alleles were investigated. In the 9beta AG group, carriers of the 9beta G allele (allele 5) were only paired with the ´Most Common Allele´ (MCA, allele1). In the N363S AG and ER22/23EK group, the variant alleles were paired with allele 1 and allele 4*

5.4.2 Cortisol Levels

Under hydrocortisone administration, cortisol levels increased significantly on both test days (day 1: $t_{(77)}$=-10.2, p=.0001; day 2: $t_{(90)}$=-11.56, p=.0001), affirming that subjects ingested the tablet.

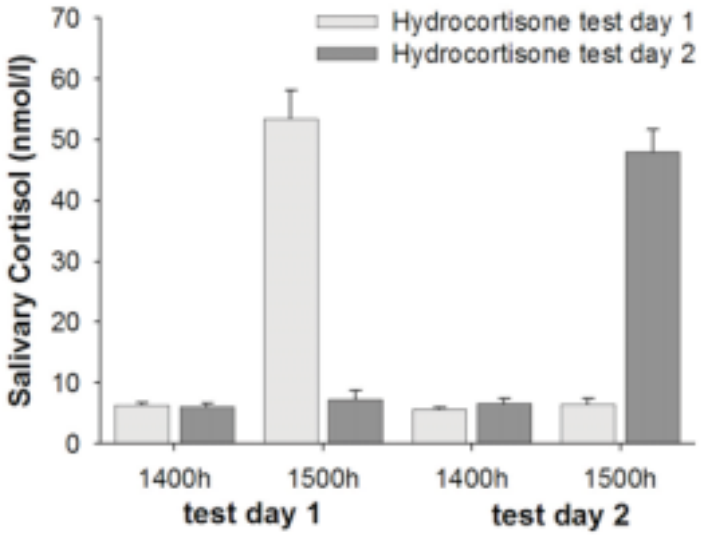

Figure 5.2: *Salivary cortisol levels before (1400h) and one hour after (1500h) treatment administration. Cortisol levels increased significantly in subjects on test days under 10mg hydrocortisone treatment, while there was no change in cortisol levels under placebo treatment*

One hour after placebo administration, cortisol concentration had not changed (day 1: t_{75}=-.78, p=.43; day 2: t_{92}=-.79, p=.43; Figure 5.2). There was no difference in cortisol levels before or after hydrocortisone or placebo administration comparing the two test days ($F_{1,132}$=.11, p=.74). In addition, no differences between genotype groups in cortisol levels before or after hydrocortisone or placebo treatment on either test day were observed ($F_{5,132}$=.56, p =.75).

5.4.3 Effects of Task Exposure Sequence and Treatment

A significant sequence effect was observed indicating better performance at test day 2 ($F_{1,156}$=76.56, p= 0.001), shown in Figure 5.3. Reaction times were on average 116.0 ± 5.7 ms or 10.4 % lower on the second test day one week after the first exposure. Therefore, subsequent analyses of treatment effects were performed with task exposure sequence as covariate.

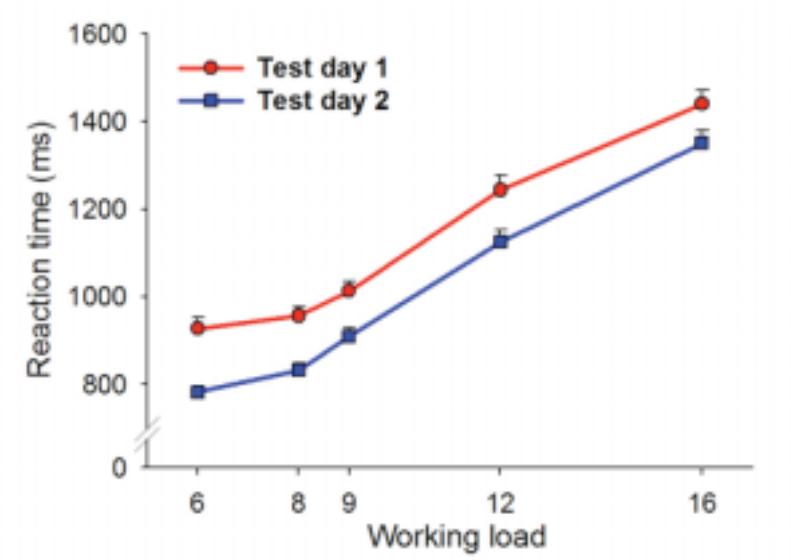

Figure 5.3: *Mean reaction times in ms in relation to working load. Subjects performed significantly better on test day 2, indicating an effect of task exposure sequence*

No influence of hydrocortisone treatment on reaction time became evident when analyzed across two test days ($F_{1,167}$=.01, p=.92; Figure 5.4). However, a significant *treatment* by *test day* effect ($F_{1,167}$=101.47, p=.0001) was revealed as indicated in Figure 5.5 a and b. On test day 1, reaction times were higher under hydrocortisone treatment whereas reaction times were lower under hydrocortisone when administrated on test day 2. When analyzed separately for test days, there was a trend towards a main effect of treatment on reaction times (day 1: $F_{1,174}$=2.34, p=.13; day 2: $F_{1,169}$=2.93, p=.09).

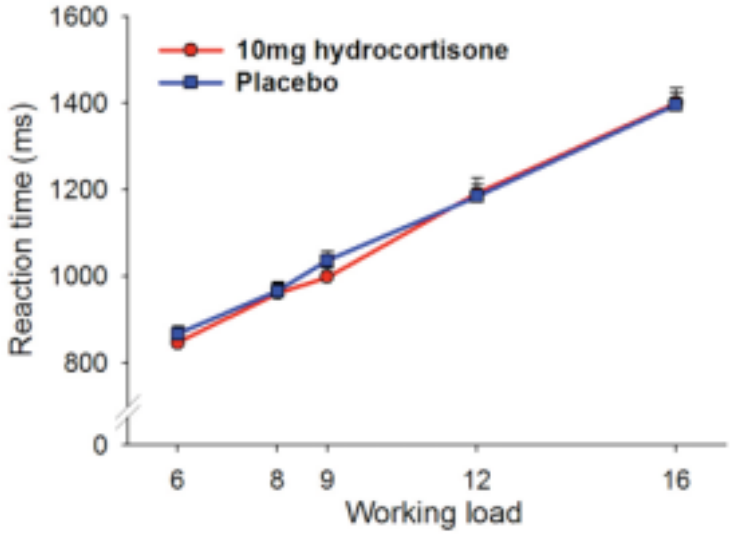

Figure 5.4: *Reaction times in relation to working load, comparing performance under hydrocortisone treatment with performance under placebo*

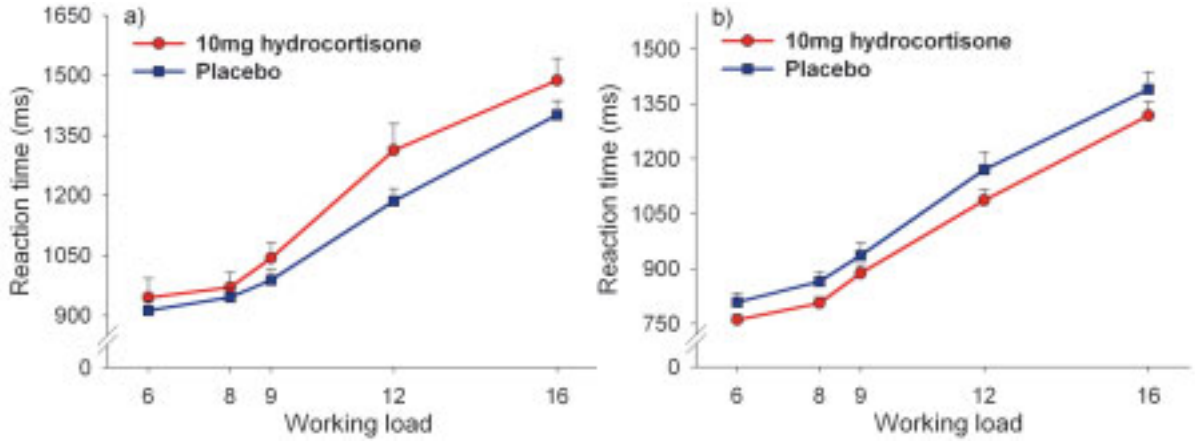

Figure 5.5: *Reaction times under different treatment conditions depicted for test day 1* ***a)*** *and test day 2* ***b)***

The magnitude of the sequence effect was identical with regard to order of treatment administration. Reaction times were 117.5 ± 21.4 ms lower on test day 2 in subjects who received hydrocortisone on test day 1 and 114.8 ± 12.1 ms lower on test day 2 in subjects who received hydrocortisone on test day 2.

Regression analysis revealed a significant positive and linear association between cortisol levels and reaction times after cortisol intake on test day 1 (R^2=.05, p=.05; Figure 5.6 a). This association was neither detected after cortisol intake on test day 2 (R^2=.001, p=.83, Figure 5.6 b) nor after placebo intake on either test day (R^2<.0001, p>.76; not shown in a Figure).

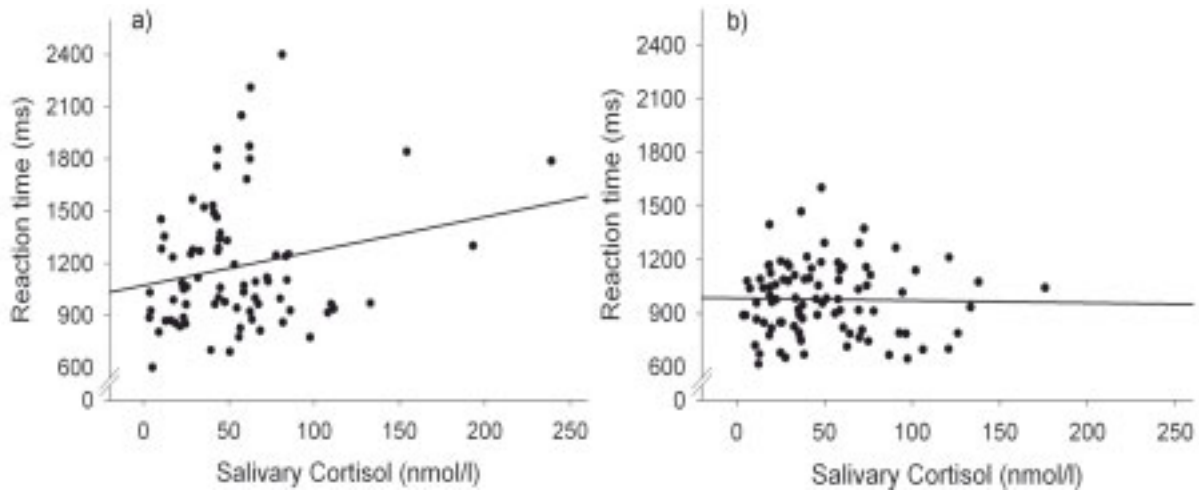

Figure 5.6: *Association between reaction times and salivary cortisol levels at 1500h in subjects under hydrocortisone treatment on* ***a)*** *test day 1 on* ***b)*** *test day 2*

Effect of genotype

The effect of task exposure sequence was similar for all genotype groups (range: 76.8 ± 4.3 – 165.4 ± 17.1 ms; interaction *genotype* by *test day*: $F_{5,156}$=.98, p=.43). In addition, no differential effect of hydrocortisone treatment according to genotype was observed, as there was no significant difference between reaction times under different treatment conditions (interaction *genotype* by *treatment*: $F_{5,149}$=1.37, p=.24).

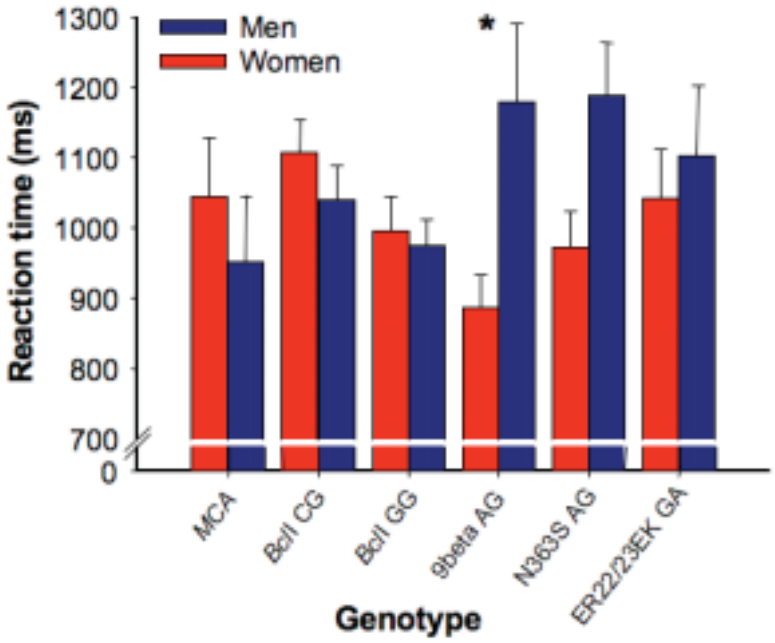

Figure 5.7: *Reaction times averaged over working loads in the GR genotype groups shown for men and women. The asterisk indicates a sex specific effect of the 9beta variant on working memory performance (*p=.02)*

Thus, reaction times were averaged over the two test days in order to analyze a general effect of GR genotype on working memory performance. Reaction times averaged over all working loads are shown for all genotype groups for men and women in Figure 5.7. For men, as indicated in Figure 5.8 a, there was substantial variability in reaction times, however, no main effect of genotype could be observed ($F_{5,67}$=1.44, p=.22). For women, a trend towards a significant main effect for genotype became evident ($F_{5,90}$=1.98, p=.08; Figure 5.8 b). 9beta AG carriers had the lowest and the *Bcl*I CG genotype had the highest reaction times for every working load (LSD Post hoc analysis: p=.004).

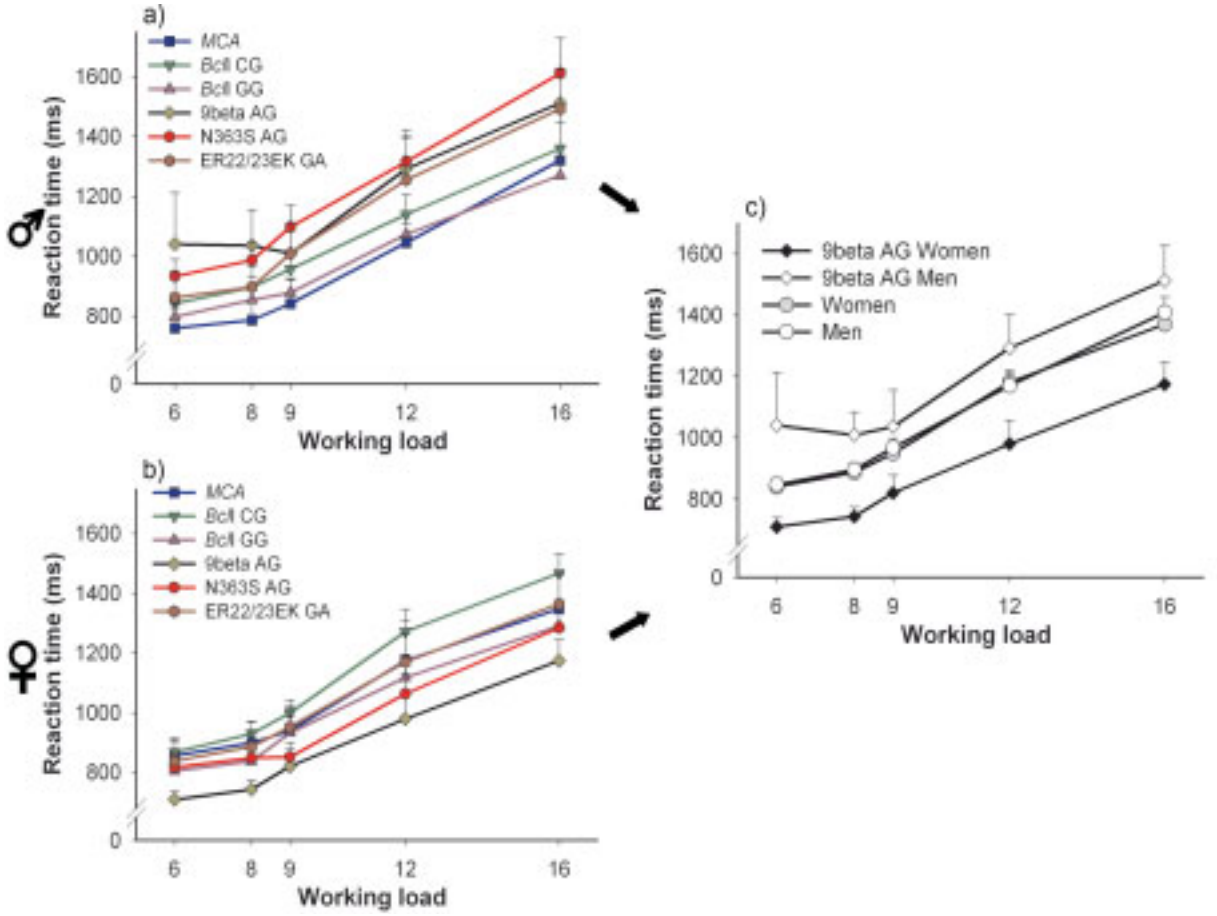

Figure 5.8: *Reaction times in relation to working load in GR genotype groups for **a)** men and **b)** women. Sex specific effect of the 9beta AG genotype is shown in part **c)**, comparing male and female 9beta AG carriers with men and women of the other genotype groups*

In addition, a significant *sex* by *genotype* association could be revealed ($F_{5,149}$=2.75, p=.02). Male 9beta AG carriers together with the N363S genotype displayed the highest reaction times while female carriers of the same genotype had the lowest reaction times. Subsequent analyses revealed a significant *sex* by *genotype*

interaction for the 9beta AG genotype ($F_{1,158}$=5.47, p=.02) in comparison with the other genotype groups and *sex* by *genotype* interaction for the N363S genotype just missed significance ($F_{1,158}$=3, p=.08). In Figure 8c, reaction times for male and female 9beta AG carriers are depicted in comparison with male and female carriers of the other groups. Differences in reaction times are evident for all working loads, the lowest delta being 189.4 ± 16.5 ms (at working load 8) and the highest 338.1 ± 41.5 ms (at working load 16).

5.5 Discussion

In order to investigate the question if GR genotype has a modulating influence on the impairing effects of cortisol administration on working memory performance, we chose a placebo-controlled, within-subject design. Using this design, the problem of sequence or training effect emerged. Although the two test sessions were separated by a one-week interval, subjects performed significantly better on the second test day. Thus, a potential treatment effect was confounded with the sequence effect, which leads to difficulties in the interpretation of the results. When analyzed across the two test sessions, no treatment effect became visible with virtually identical reaction times. Separate analysis for test days can lead to conclusive interpretations only for the first test day when subjects are test naïve. There was a trend for an impairing effect of hydrocortisone on working memory performance and correlational analysis indicate that high cortisol levels are associated with impaired performance. This is in line with reports by Lupien et al. (1999) reporting impairment of WM performance under high doses of hydrocortisone infusion. Impairments, however, where only seen at doses of 600µg/kg/hr, which corresponds 88.9 mg of hydrocortisone, a dose much higher than the 10mg used in the present study. This might explain why only slight impairments in performance were observed.

Interpretation of treatment effects on test day 2 is confounded with the sequence effect and allows speculation only. The fact that the group under hydrocortisone treatment on test day 2 performed better than the placebo group, opposite to test day 1, is most likely due to the fact that this group had performed better on test day 1, having received placebo. The magnitude of the sequence effect was identical for both groups, i.e. regardless of treatment on test day 2, performance ameliorated identically. This allows the speculation that hydrocortisone treatment only impairs working memory performance when no prior learning of the task has occurred. This

is supported by the observation that there was no association of cortisol levels with reaction times in subjects who had already performed the task, in contrast to the linear positive association in test naïve subjects. It has been shown that the impairing effects of cortisol administration on declarative and working memory performance depend on concurrent noradrenergic activation in the basolateral amygdala (Elzinga and Roelofs 2005; Roozendaal 2002; Roozendaal et al 2003; Roozendaal et al 2004). Possibly, the fact that the task and the test facility was novel to the subjects on test day 1 might have led to activation of the stress system, i.e. activation of the sympathetic nervous system and HPA axis, since situations characterized by novelty, unpredictability and ego-involvement are known to reliably stimulate the stress system. Thus, subjects who received hydrocortisone and experienced the task for the first time might have had an additional increase in cortisol levels thereby sufficiently elevating cortisol levels to cause impairments in performance. On test day 2, subjects virtually experienced the identical situation. Thus, no activation of the stress system would be expected and therefore no impairment effect of exogenous cortisol on WM performance was observed.

Genotype effects

Since no impairing effect of hydrocortisone could be observed within subjets, the question if GR genotype modulates the effects of acute hydrocortisone treatments on WM performance could not be answered by this study. Analysis of performance of test day 1 only - without the confounding sequence effects - is not feasible, since the between-subjects comparison, together with the small group sizes, would lead to losses in statistical power. On a descriptive level, all genotype groups performed about equally worse under hydrocortisone treatment compared to the placebo condition. Therefore, performance on both test days was averaged in order to identify a general effect of GR genotype on WM performance independent of treatment or sequence effects (given that the sequence effect was nearly identical between the genotype groups).

We observed a significant and sex specific effect of the 9beta AG variant on WM performance. In women, 9beta AG carriers had lower reaction times than the other women, while in men, the same genotype was among the groups with the highest reaction times. A sex specific genetic architecture of quantitative traits has been observed for a number of measures (see Weiss et al 2006 for review) and we have observed sex specific effects of the 9beta variant with regard to HPA axis regulation. While no differences were observed in women, male 9beta AG carriers had

significantly higher ACTH and total serum cortisol responses following a psychosocial stress protocol and, in addition, significantly higher ACTH and salivary cortisol levels were observed after Dexamethasone suppression, indicating a relative GC resistance (Kumsta et al in prep). This was in accordance with previous *in vivo* and *in vitro* studies. Functional analyses revealed a stabilizing effect of this polymorphism on GRbeta mRNA *in vitro*, leading to enhanced expression of GRbeta protein (Derijk et al 2001). GRbeta protein is unable to bind ligand and acts as a dominant negative inhibitor of GRalpha (Charmandari et al 2005; Yudt et al 2003).

The question arises if alterations in GC sensitivity can explain part of the differences in WM performance between the genotype groups. An inverted-U-shape function between circulating levels of corticosteroids and cognitive performance has been suggested for hippocampus dependent declarative memory (De Kloet et al 1998). This function has been interpreted by DeKloet et al. (1999) in line with the hypothesis that the ratio of corticosteroid receptor occupancy, i.e. mineralocorticoid and glucocorticoid receptors, is a crucial determinant of cognitive performance. Results from electrophysiological studies showed that basal levels of corticosteroids are needed for effective hippocampal long-term potentiation (LTP) while higher levels impair LTP. Thus, it was hypothesized that when circulating levels of corticosteroids are significantly decreased or increased, resulting in low MR/GR occupancy ratio, cognitive impairments will result. Cognitive function can be enhanced, on the other hand, when most MRs and only part of the GRs are activated, reflecting high MR/GR ratio. Circulating levels of cortisol are determined by a number of factors, including time of day, actual stress exposure and genetic factors. The effects of circulating cortisol depend on functional characteristics of the GR and the investigated variants are known to impact on GC sensitivity. Endogenous alterations in GC signaling caused by GR polymorphisms might in part explain the observed differences in WM performance.

One limitation of our study is that no tests controlling for arousal-vigilance or general cognitive abilities were performed.

Results from twin studies have revealed heritability estimates for cognitive abilities, including speed of processing and memory, of approximately 50% (McClearn et al 1997), indicating that naturally occurring genetic variations have an important impact on cognitive performance. For instance, deQuervain et al. (2003) have reported that a functional genetic variant of the 5HT2a receptor affects declarative memory function. Furthermore, genetic variation in COMT has been linked to inefficient

working memory performance (Meyer-Lindenberg et al 2006). Our results indicate that polymorphisms of the GR gene might be one additional factor explaining part of the variance attributable to genetic factors in working memory performance.

5.6 References

Baddeley A (1986): *Working memory*. Ney York: Oxford University Press.

Belanoff JK, Gross K, Yager A, Schatzberg AF (2001): Corticosteroids and cognition. *J Psychiatr Res* 35:127-145.

Charmandari E, Chrousos GP, Ichijo T, Bhattacharyya N, Vottero A, Souvatzoglou E, Kino T (2005): The human glucocorticoid receptor (hGR) beta isoform suppresses the transcriptional activity of hGRalpha by interfering with formation of active coactivator complexes. *Mol Endocrinol* 19:52-64.

de Kloet ER, Oitzl MS, Joels M (1999): Stress and cognition: are corticosteroids good or bad guys? *Trends Neurosci* 22:422-426.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998): Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 19:269-301.

de Quervain DJ, Henke K, Aerni A, Coluccia D, Wollmer MA, Hock C, et al (2003): A functional genetic variation of the 5-HT2a receptor affects human memory. *Nat Neurosci* 6:1141-1142.

Derijk RH, Schaaf MJ, Turner G, Datson NA, Vreugdenhil E, Cidlowski J, et al (2001): A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol* 28:2383-2388.

Diamond A (1988): Abilities and neural mechanisms underlying AB performance. *Child Dev* 59:523-527.

Dolan RJ, Fletcher PC (1997): Dissociating prefrontal and hippocampal function in episodic memory encoding. *Nature* 388:582-585.

Dressendorfer RA, Kirschbaum C, Rohde W, Stahl F, Strasburger CJ (1992): Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. *J Steroid Biochem Mol Biol* 43:683-692.

Elzinga BM, Roelofs K (2005): Cortisol-induced impairments of working memory require acute sympathetic activation. *Behav Neurosci* 119:98-103.

Fuster JM (1989): *The Prefrontal Cortex: Anatomy, Physiology and Neuropsychology of the Frontal Lobe.* New York: Raven Press.

Gold PW, Drevets WC, Charney DS (2002): New insights into the role of cortisol and the glucocorticoid receptor in severe depression. *Biol Psychiatry* 52:381-385.

Het S, Ramlow G, Wolf OT (2005): A meta-analytic review of the effects of acute cortisol administration on human memory. *Psychoneuroendocrinology* 30:771-784.

Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, et al (1998): A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *J Clin Endocrinol Metab* 83:144-151.

Kirschbaum C, Wolf OT, May M, Wippich W, Hellhammer DH (1996): Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults. *Life Sci* 58:1475-1483.

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, et al (1997): Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. *Hum Genet* 99:663-668.

Kumsta R, Entringer S, Koper JW, Van Rossum EF, Hellhammer DH, Wüst S (in prep): Sex specific associations between common glucocorticoid receptor gene variants and hypothalamus-pituitary-adrenal axis responses to psychosocial stress.

Lupien S, Lecours AR, Lussier I, Schwartz G, Nair NP, Meaney MJ (1994): Basal cortisol levels and cognitive deficits in human aging. *J Neurosci* 14:2893-2903.

Lupien S, Richter R, Risch SC, Mirow A, Gillin JC, Hauger RL (1995): Time course of the corticosteroid-dopaminergic interaction during metyrapone and dexamethasone administration. *Psychiatry Res* 58:23-35.

Lupien SJ, Gillin CJ, Hauger RL (1999): Working memory is more sensitive than declarative memory to the acute effects of corticosteroids: a dose-response study in humans. *Behav Neurosci* 113:420-430.

Lupien SJ, McEwen BS (1997): The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Res Brain Res Rev* 24:1-27.

Lupien SJ, Wilkinson CW, Briere S, Menard C, Ng Ying Kin NM, Nair NP (2002): The modulatory effects of corticosteroids on cognition: studies in young human populations. *Psychoneuroendocrinology* 27:401-416.

McClearn GE, Johansson B, Berg S, Pedersen NL, Ahern F, Petrill SA, Plomin R (1997): Substantial genetic influence on cognitive abilities in twins 80 or more years old. *Science* 276:1560-1563.

McEwen BS, De Kloet ER, Rostene W (1986): Adrenal steroid receptors and actions in the nervous system. *Physiol Rev* 66:1121-1188.

McEwen BS, Sapolsky RM (1995): Stress and cognitive function. *Curr Opin Neurobiol* 5:205-216.

Meyer-Lindenberg A, Nichols T, Callicott JH, Ding J, Kolachana B, Buckholtz J, et al (2006): Impact of complex genetic variation in COMT on human brain function. *Mol Psychiatry* 11:867-877, 797.

Miller SA, Dykes DD, Polesky HF (1988): A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.

Oitzl MS, Reichardt HM, Joels M, de Kloet ER (2001): Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. *Proc Natl Acad Sci U S A* 98:12790-12795.

Owen AM, Downes JJ, Sahakian BJ, Polkey CE, Robbins TW (1990): Planning and spatial working memory following frontal lobe lesions in man. *Neuropsychologia* 28:1021-1034.

Petrides M, Milner B (1982): Deficits on subject-ordered tasks after frontal- and temporal-lobe lesions in man. *Neuropsychologia* 20:249-262.

Roozendaal B (2002): Stress and memory: opposing effects of glucocorticoids on memory consolidation and memory retrieval. *Neurobiol Learn Mem* 78:578-595.

Roozendaal B, Griffith QK, Buranday J, De Quervain DJ, McGaugh JL (2003): The hippocampus mediates glucocorticoid-induced impairment of spatial memory retrieval: dependence on the basolateral amygdala. *Proc Natl Acad Sci U S A* 100:1328-1333.

Roozendaal B, Hahn EL, Nathan SV, de Quervain DJ, McGaugh JL (2004): Glucocorticoid effects on memory retrieval require concurrent noradrenergic activity in the hippocampus and basolateral amygdala. *J Neurosci* 24:8161-8169.

Russcher H, Smit P, van den Akker EL, van Rossum EF, Brinkmann AO, de Jong FH, et al (2005): Two Polymorphisms in the Glucocorticoid Receptor Gene Directly Affect Glucocorticoid-Regulated Gene Expression. *J Clin Endocrinol Metab*.

Sarrieau A, Dussaillant M, Sapolsky RM, Aitken DH, Olivier A, Lal S, et al (1988): Glucocorticoid binding sites in human temporal cortex. *Brain Res* 442:157-160.

Smith EE, Jonides J, Marshuetz C, Koeppe RA (1998): Components of verbal working memory: evidence from neuroimaging. *Proc Natl Acad Sci U S A* 95:876-882.

Sternberg S (1966): High-speed scanning in human memory. *Science* 153:652-654.

van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, et al (2002): A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. *Diabetes* 51:3128-3134.

Van Rossum EF, Lamberts SW (2004): Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. *Recent Prog Horm Res* 59:333-357.

Weiss LA, Pan L, Abney M, Ober C (2006): The sex-specific genetic architecture of quantitative traits in humans. *Nat Genet* 38:218-222.

Wolf OT (2003): HPA axis and memory. *Best Pract Res Clin Endocrinol Metab* 17:287-299.

Yudt MR, Jewell CM, Bienstock RJ, Cidlowski JA (2003): Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* 23:4319-4330.

CHAPTER 6

The Influence of CBG on the Activity of the Hypothalamus-Pituitary-Adrenal Axis in Men, and in Women Usin g Oral Contraceptives

6.1 Summary

Background: The hypothalamus-pituitary-adrenal (HPA) axis is vital for an organisms´ response to physiological and psychological stress. Cortisol, secreted upon activation of the HPA axis, regulates or impacts on physiological systems throughout the organism. The cellular and organismal responses to cortisol are not solely regulated by the actual levels of circulating glucocorticoid as a number of other factors contribute to and modify responses to cortisol, including the level of circulating corticosteroid binding globulin (CBG). A major part of circulating cortisol is bound to CBG and only the unbound fraction is thought to be metabolically active.
Methods: The aim of the present study was to examine the effect of CBG on hormonal responses following psychosocial stress and pharmacological stimulation (1µg $ACTH_{1-24}$ stimulation, 0.25 mg Dexamethasone suppression test). In addition, a salivary cortisol diurnal profile was assessed. This study investigated 115 women using oral contraceptives and 93 medication-free men.
Results: In women, CBG levels correlated negatively with ACTH and salivary cortisol and positively with total cortisol levels following the TSST. In men, positive correlations were observed between CBG and ACTH and total cortisol levels following the TSST. In response to $ACTH_{1-24}$ stimulation, positive correlations between CBG and total cortisol levels were observed in women and in men, while there was a negative correlation between CBG and salivary cortisol in women and no association in men. Following Dex suppression, negative correlation between salivary cortisol and CBG was revealed in women, while no association could be detected in men. No influence was detected on basal cortisol levels throughout the day neither in men nor in women.
Conclusions: CBG is an important regulatory element of HPA axis response patterns; therefore, CBG levels have to be taken into account as a potential modifier of ACTH and cortisol responses to psychosocial and pharmacological stimulation.

6.2 Introduction

Activation of the hypothalamus-pituitary-adrenal (HPA) axis by physiological or psychological stress results in the secretion of cortisol from the adrenal gland. Cortisol, the most important glucocorticoid in humans, plays a crucial role in energy mobilization, regulates or impacts on other physiological systems and also influences affective and cognitive functions. Besides these processes, cortisol is important for functioning of the HPA axis itself by imposing negative feedback on hippocampal and hypothalamic sites as well as on level of the pituitary, thereby terminating the stress response (de Kloet et al 2005). The cellular and organismal responses to cortisol are not solely regulated by the actual levels of circulating glucocorticoid, as a number of other factors contribute to and modify responses to cortisol. Tissue specific glucocorticoid and mineralocorticoid receptor distribution and receptor number, the efficiency of processes from receptor activation to DNA binding and the presence and activity of enzymes such as 11beta-hydroxysteroid dehydrogenase (11betaHSD) I and II influence the efficacy of the glucocorticoid signal (Bamberger et al 1996). Another important factor is the level of the circulating corticosteroid binding globulin (CBG). A major part of circulating cortisol is bound to plasma proteins, mostly to CBG (Hammond 1990; Rosner 1990; Siiteri et al 1982). According to the free hormone hypothesis (Ekins 1990; Westphal 1983), the bound fraction of cortisol is unavailable to tissues and only the unbound fraction (about 10% of total cortisol) is thought to be metabolically active. Thus, CBG levels determine the actual amount of cortisol available to the organism. It has been shown that CBG synthesis is regulated by GCs (Cole et al 1999) and that CBG concentration is influenced by circulating gonadal steroids, e.g. in pregnancy (Kajantie and Phillips 2006). Especially the use of ethinyl-estradiol containing oral contraceptives (OC) has been shown to stimulate CBG synthesis (Fujimoto et al 1986; Wiegratz et al 1995; Wiegratz et al 2003). Investigations of OC using women have revealed changes in salivary cortisol response patterns to psychosocial (Kirschbaum et al 1995) and pharmacological stimulation. In comparison to women in the luteal phase, OC using women displayed a blunted salivary cortisol response to the Trier Social Stress Test (TSST), while no differences were observed in ACTH or total serum cortisol response patterns. Following $ACTH_{1-24}$ stimulation, OC users had the lowest responses in salivary cortisol compared with women in either luteal or follicular phase while no group

differences were observed in total serum cortisol (Kirschbaum et al 1999). It has been hypothesized that the observed lower salivary cortisol levels might be due to higher levels of CBG resulting from OC intake. However, the impact of CBG levels on HPA axis responses has not yet been systematically investigated. The aim of the present study, therefore, was to examine the effect of CBG on hormonal responses following psychosocial stress, a 1 µg Synacthen ($ACTH_{1-24}$) stimulation and a low-dose (0.25 mg) Dexamethasone suppression test. Moreover, a salivary cortisol diurnal profile was assessed. This study investigated women using oral contraceptives, where high levels and a wide range of CBG could be expected. In addition, a group of medication-free men was studied, where no exogenous factors influenced CBG levels, in order to assess if effects of CBG become already visible with lower levels and a smaller range than observed in OC using women.

6.3 Methods

6.3.1 Subjects

For the present study, 115 women and 93 men were recruited. All females were using ethynil-estradiol containing oral contraceptives (OC) and all males were medication free. Prior to the first experimental session, the absence of acute or chronic diseases was confirmed in a medical exam. The study protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants.

6.3.2 Experimental Protocol

Participants reported to the laboratory three times. Test days one and two were separated by a one-week interval, and subjects reported to the laboratory in the afternoon. Forty-five minutes before the challenge tests, an iv catheter was inserted in an antecubital vein and kept patent with a lock. Each participant was exposed to two different stimulation procedures at identical times across test days between 1500 and 1600 h. On the first day, volunteers received an iv injection of 1 µg Synacthen (Novartis, Nuernberg, Germany, low-dose $ACTH_{1-24}$ stimulation test). On the second test day subjects were exposed to the Trier Social Stress Test (TSST), which consists of a free speech and a mental arithmetic task of 15 min duration performed in front of a panel and a camera. This stress protocol has been found to induce significant cortisol, ACTH, and cardiovascular responses at the first exposure in 70-80% of all subjects. On test day three, a low-dose Dexamethasone suppression test

(DST) was performed. Participants were instructed to ingest 0.25 mg Dexamethasone (Par Pharmaceutical, Spring Valley, USA) at 2300 h, and they reported to the laboratory the next morning between 0800 and 0830 h. The time interval between test day three and test days one and two was at least 48h. In addition, subjects collected saliva samples at home. The cortisol awakening rise (CAR) was assessed on the morning after Dexamethasone ingestion and salivary samples for a diurnal cortisol profile were obtained on a separate test day.

6.3.3 Blood and Saliva Sampling

On the day of the $ACTH_{1-24}$ stimulation test, blood samples were collected 2 min before and 15, 30, 45, 60, 75, and 120 min after drug injection to assess total cortisol levels, and saliva samples were obtained using Salivette sampling devices (Sarstedt, Nuembrecht, Germany) at the same time points as blood samples, and in addition at 90 and 105 min after the injection for the assessment of salivary cortisol levels. On the TSST day, saliva, serum blood and EDTA blood samples were collected 2 min before and 1, 10, 20, 30 and 90 min after cessation of the TSST to assess total cortisol and ACTH levels. Additional saliva samples for the assessment of salivary cortisol were obtained at 45 and 60 min using Salivette sampling devices (Sarstedt, Nuembrecht, Germany). Blood samples obtained before the $ACTH_{1-24}$ stimulation test and before the TSST were used to measure CBG levels. The day after Dexamethasone ingestion, subjects were instructed to collect saliva samples immediately after awakening and 30, 45 and 60 minutes thereafter. Between 0800 and 0830 h, one blood sample was obtained to assess Dexamethasone levels. For the assessment of the diurnal cortisol profile, subjects collected saliva samples at half hour intervals from 0800h to 1800h. EDTA blood samples were immediately stored on ice and centrifuged within 30 min at 2000 x g and 4° C for 10 min. EDTA plasma was divided into aliquots and stored at -80° C until analysis. Serum blood samples stood at room temperature for 30 min before they were processed as the plasma samples and stored at -20°C until analysis. Saliva samples were kept at room temperature throughout one test session and then stored at -20°C. After thawing for biochemical analysis, samples were centrifuged at 2000 x g at 10°C for 10 min.

6.3.4 Biochemical Analyses

Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendorfer et al 1992). Total cortisol concentrations were measured in serum blood with an ELISA (IBL, Hamburg,

Germany). ACTH was measured in EDTA plasma with a chemiluminescence immunoassay (Nichols institute, Bad Nauheim, Germany). Plasma Dexamethasone was assessed with an in-house RIA at the Institute of Pharmacology, University of Heidelberg. CBG was measured with a RIA (IBL, Hamburg, Germany). Interassay and intraassay coefficients of variance were below 10% and 12%, respectively, for all analyses.

6.3.5 Statistical Analyses

All data were log transformed before analysis to yield unskewed outcome variables and all analyses were computed separately for men and women. The repeated measures effect *time* for endocrine responses to the $ACTH_{1-24}$ stimulation, TSST exposure and the DST and for salivary cortisol levels in the diurnal profile was assessed with a General Linear Model (GLM) procedure. In order to estimate the impact of CBG, this variable was included in a subsequent model. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. One-way ANOVAs were performed to compare mean serum CBG levels between men and women. Correlation analyses were performed on the relationship between CBG levels and hormonal responses expresses as area under the curve with respect to ground (AUC_G , Pruessner et al 2003). All results shown are the mean ± SEM.

6.4 Results

6.4.1 CBG Levels

As shown in Table 6.1, CBG levels differed significantly between men and women ($F_{1,206}$=337.49, p =.0001). For graphical illustration of the effect of the continuous measure CBG, median groups for this variable were calculated (Table 6.2) for women and men.

	Men	Women	F	p
N	93	115		
Age	25,7 ±.41	24,7 ±.36	3,914	.049
BMI	25,5 ±.37	23,5 ±.40	13,879	.0001
CBG	43.32 ± .96	105.00 ± 2.19	337.49	.0001

Table 6.1*: Age, BMI and CBG levels in female and male subjects*

Women	**N**	**CBG mean (µg/ml)**
Below median	54	79.49 ± 2.98
Above median	61	128.59 ± 1.88
Men		
Below median	47	35.77 ± .84
Above median	46	51.05 ± .66

Table 6.2: *CBG levels in the median groups in men and women*

6.4.2 TSST

Exposure to the TSST lead to significant increases in ACTH, total cortisol and salivary cortisol levels for both men and women ($F>143.0$, $p<.0001$ for all analytes) Men showed significantly higher increases in ACTH (main effect *sex*: $F_{1,208}=116.2$, $p=.0001$; interaction *sex* x *time*: $F_{3.1,\ 641.7}=9.3$; $p=.0001$) and salivary cortisol (main effect *sex*: $F_{1,208}=15.21$, $p=.0001$; interaction *sex* x *time*: $F_{3.0,\ 649.2}=27.9$, $p=.0001$), while women displayed significantly higher levels of total cortisol (main effect *sex*: $F_{1,208}=54.5$, $p=.0001$; interaction *sex* x *time*: $F_{2.7,\ 553.3}=37.74$, $p=.0001$).

Influence of CBG Levels - TSST

Significant associations between CBG levels and hormonal responses following the TSST were revealed. For women, TSST reactions are shown for median of CBG levels in Figure 6.1 a-c.

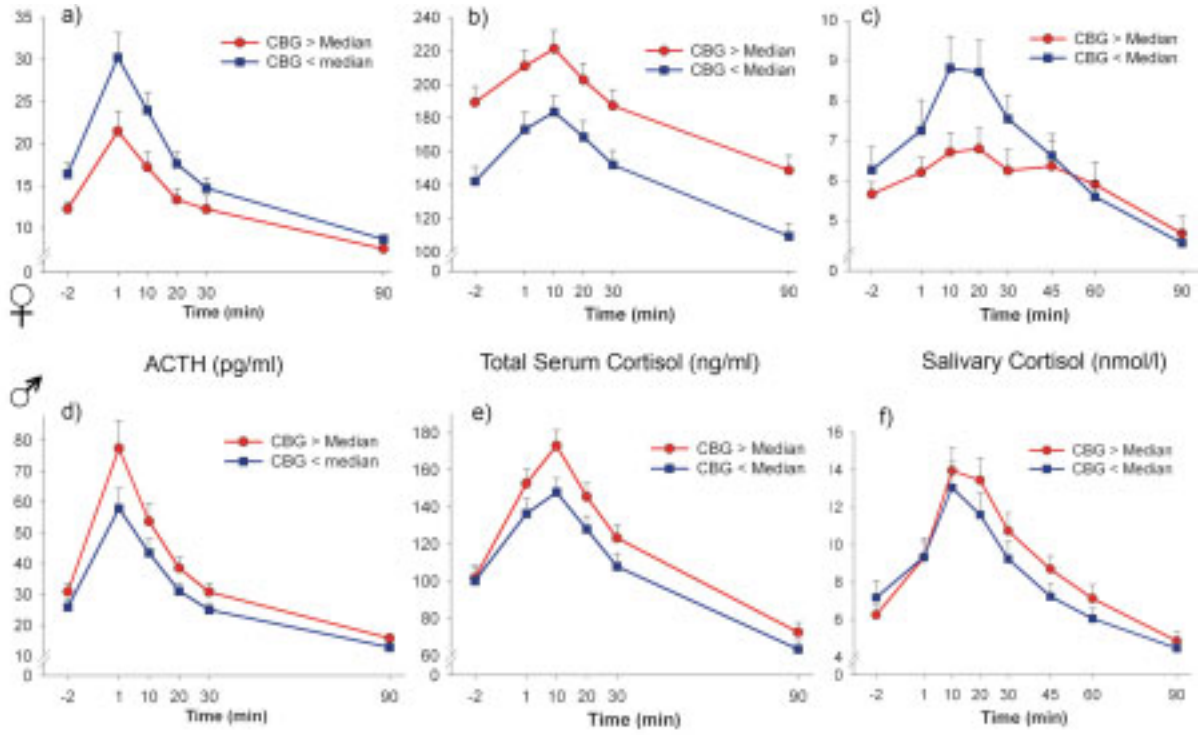

Figure 6.1: *Hormonal responses following the TSST in women and in men depicted for median groups of CBG. Figures a-c show ACTH, total serum cortisol and salivary cortisol levles in women and Figures d-f show ACTH, total serum cortisol and salivary cortisol levels in men*

In females, the group above the median displayed lower peak concentrations of ACTH following psychosocial stress, whereas the group with lower CBG levels mounted higher responses, resulting in a significant main effect ($F_{1,104}$=6.13, p=.01). No significant interactions were detected ($F_{1.9,205.9}$=1.75, p=.12). Total and salivary cortisol levels were inversely related to CBG levels. Low CBG levels were associated with low total cortisol and high salivary cortisol levels, whereas high CBG levels resulted in high concentrations of total and low concentrations of salivary cortisol (Main effect CBG for total cortisol: $F_{1,108}$=18.8, p=.0001; interaction *time* x *CBG* for total cortisol: $F_{3.2,346.9}$=.39, p=.78). For salivary cortisol responses, a significant *time* x *CBG* interaction ($F_{1,111}$=4.27, p= .008) was observed while the main effect *CBG* was not significant ($F_{1,111}$=.59, p=.45). Correlation analyses revealed that CBG levels correlated negatively with ACTH (r=.-.22, p=.02) and salivary cortisol levels (ns) expressed as AUC. In contrast, there was a positive correlation between CBG and the AUC of total cortisol (r=.38, p=.0001).

In men, as indicated in Figure 6.1 d, ACTH levels were higher with high CBG levels. A main effect *CBG* was observed for ACTH responses to the TSST ($F_{1,88}$=4.28,

p=.04) and the *time* by *CBG* effect just missed significance ($F_{1.4,120.9}$=2.8, p=.08). In addition, a main effect *CBG* was shown for total cortisol responses ($F_{1,88}$=5.73, p=.02; Figure 6.1 e), and there was a trend towards a *time* by *CBG* interaction effect ($F_{2.9,256}$=2.43, p=.07). Although men with lowest CBG levels displayed the smallest increase in salivary cortisol, shown in Figure 6.1 f, no significant main effect or interaction effect was revealed (main effect *CBG*: $F_{1,86}$=1.9, p=.17; interaction *sex* x *time*: $F_{2.9,\ 245.1}$=.98 ; p=.40). When hormonal responses were expressed as AUC, significant positive correlations were observed between CBG and ACTH levels (r=.22, p=.03) as well as total cortisol levels (r=.25, p=.02). For salivary cortisol, a trend towards a positive correlation was detected (r=.15, p=.15).

6.4.3 Synacthen ($ACTH_{1-24}$) Stimulation Test

As indicated in Figure 6.2 a and b, differences in hormonal responses following Synacthen stimulation became evident in women for total and salivary cortisol responses. Total cortisol responses incremented with rising levels of CBG (*time* by *CBG* effect: $F_{2.8,293.2}$=10.38, p=.0001; main effect *CBG*: $F_{1,105}$=49.88, p=.0001) Regarding response patterns for salivary cortisol, the group with lower CBG levels showed higher responses (*time* by *CBG*: $F_{3.2,350,2}$=9.73, p= .0001; main effect *CBG*: $F_{1,110}$=.46, p=.50). Correlational analyses with CBG levels and total cortisol expressed as AUC revealed a positive association of CBG and total cortisol levels (r=.58, p=.0001) and a negative, but not significant, association with salivary cortisol levels (r=-.06, p=.49).

In men, a significant *time* by *CBG* effect ($F_{2.8,252.5}$=3.44; p=.02) for total cortisol responses became evident while no main effect was detected (*CBG*: $F_{1,89}$=1.21, p=.27). The group below the median displayed lower peak concentration than the group with higher CBG levels (Figure 6.2 c). There was no influence of CBG levels on salivary cortisol reactions in men, shown in Figure 6.2 d (main effect *CBG*: $F_{1,90}$=.6, p=.44; *time* by *CBG*: $F_{2.4,216.1}$=1.07, p=.35) and regression analyses did not reveal any significant associations for total or salivary cortisol levels expressed as AUC with CBG.

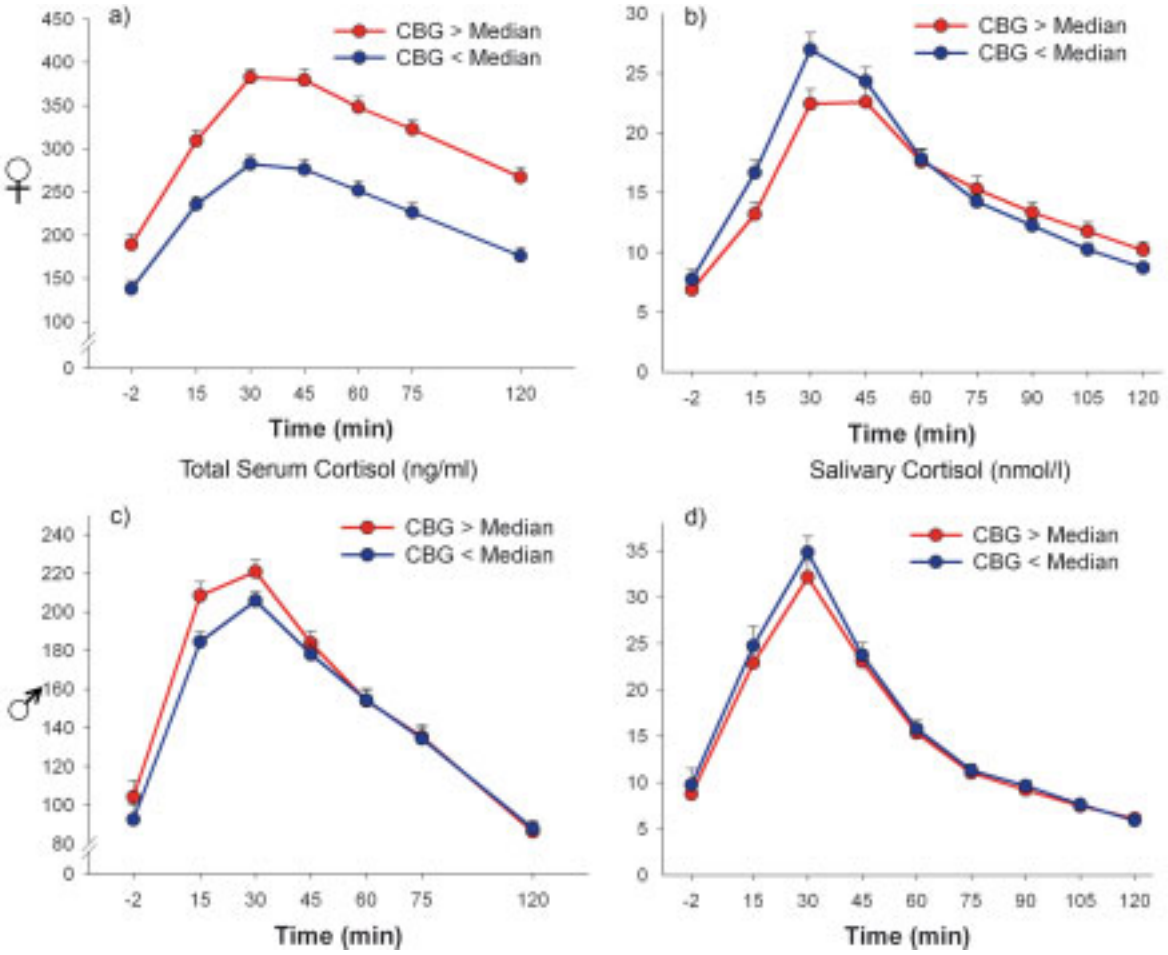

Figure 6.2: *Total serum and salivary cortisol responses following 1µg $ACTH_{1-24}$ stimulation in women and in men depicted for median groups of CBG. Figures a) and b) show total serum cortisol and salivary cortisol levles in women and Figures c) and d) show total serum cortisol and salivary cortisol levels in men*

6.4.4 Dexamethasone Suppression Test

For the cortisol awakening rise after the Dexamethasone Suppression Test, significant effects of CBG became visible for women (*time* by *CBG*: $F_{1.8,179.9}$=4.97, p=.01; main effect *CBG*: $F_{1,102}$=11.57, p=.0001), but not for men (Figure 6.3 a and b). Higher concentrations of CBG were related to a more pronounced suppression in the first hour after awakening in women. CBG levels correlated negatively with salivary cortisol expressed as AUC in women (r=-.30, p=.002). In men, no association was observed (*time* by *CBG*: $F_{2.1,168.2}$=.99, p=.38; main effect *CBG*: $F_{1,80}$=.22, p=.64).

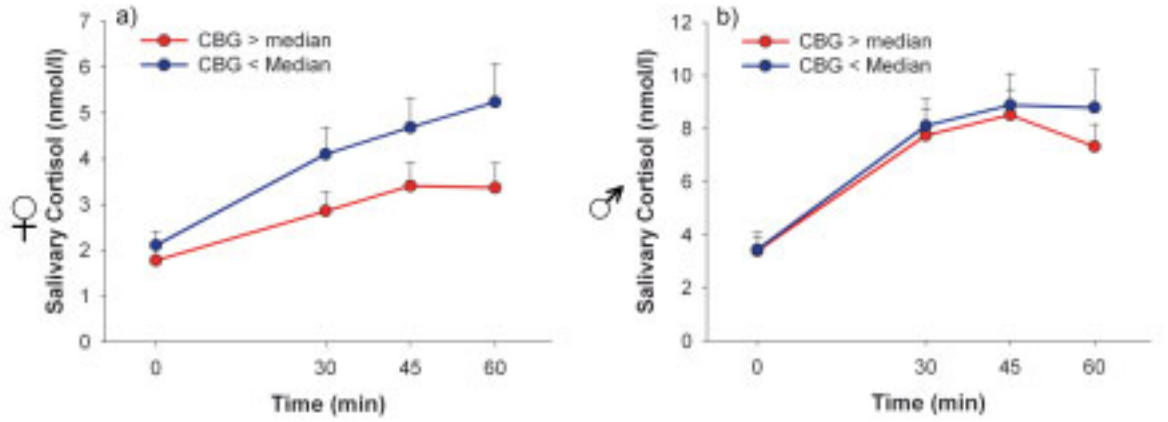

Figure 6.3: *Salivary cortisol levels in the first hour after awakening after 0.25 mg Dexamethasone ingestion in a) women and b) men; shown for median groups of CBG*

6.4.5 Diurnal Profile

No influence of CBG on salivary cortisol levels in the diurnal profile could be detected neither in women nor in men (Figure 6.4 a and b). On a descriptive level, it is noteworthy that in women the group with higher CBG levels had slightly higher salivary cortisol levels in the morning than the group with lower levels, which is contrast to the lower levels observed with high CBG after stimulation.

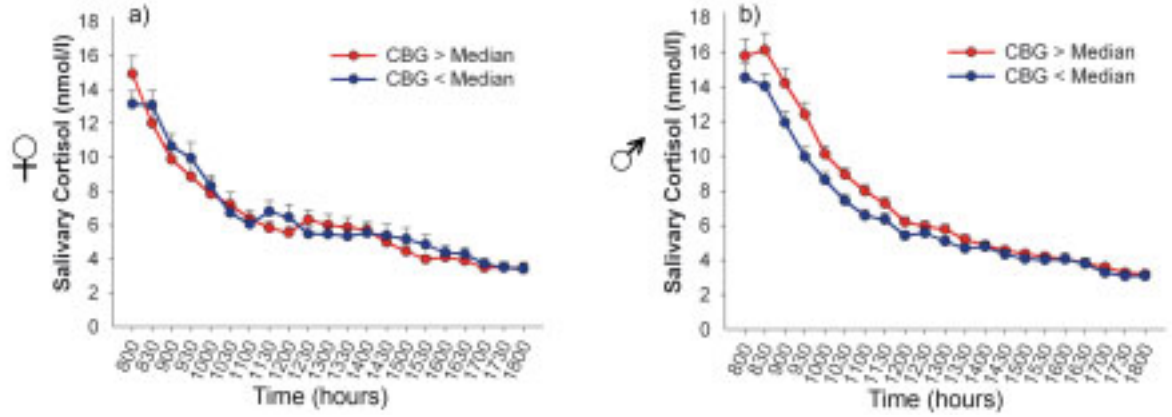

Figure 6.4: *Salivary cortisol diurnal profile obtained at 30-minute intervals between 0800 and 1800h. Shown for median groups of CBG in a) women and b) men*

6.5 Discussion

It is well documented that women using OC display a blunted salivary cortisol response following psychosocial and pharmacological stimulation and it was suggested that this was due to the CBG-enhancing effect of ethinyl-estradiol (Kirschbaum et al 1999; Kirschbaum et al 1995). The influence of CBG levels,

however, was not directly related to hormonal responses in previous studies and OC using women were compared as a group with other women in different phases of their menstrual cycle and with men. Therefore, two questions were addressed in the present study. First, can CBG concentrations explain variation of hormonal responses to HPA axis stimulation within the group of OC using women? Second, is the impact of CBG as a regulatory element of HPA axis responses limited to women with exogenously up-regulated CBG levels, or does CBG also impact on hormonal responses to HPA axis stimulation in men with lower levels and a narrower range of CBG?

Addressing the first questions, the results indicate that CBG influences hormonal responses following $ACTH_{1-24}$ stimulation, Dex suppression and psychosocial stimulation in OC using women. In contrast to the rather prominent differences in response to stimulation, no impact of CBG on basal cortisol measures assessed by a salivary cortisol diurnal profile was observed in women. Following psychosocial stress in OC using women, as could be expected, salivary cortisol responses were clearly lower and total serum cortisol levels were distinctly higher with high CBG levels. In addition, a negative association of CBG with ACTH responses to the TSST was observed. The diminished ACTH responses, being speculative, might reflect a compensatory down-regulation of CRH receptors on pituitary level. In the course of OC intake and the concomitant increase of CBG, a larger fraction of free cortisol will be bound, resulting in a signal to increase cortisol secretion. This process will continue until CBG becomes saturated. The consequences will be higher levels of total cortisol levels, as observed in our study, and possibly a down-regulation of CRH receptors on the pituitary in light of an increased CRH signal, explaining lower ACTH increases with high CBG levels.

In accordance with previous findings, there was a clear positive correlation with CBG and total cortisol levels (Dhillo et al 2002) and a negative correlation with CBG and salivary cortisol levels following $ACTH_{1-24}$ stimulation. Results from the Dex test indicate lower salivary cortisol levels associated with higher CBG concentrations. These differences might be interpreted as a more pronounced suppression; however, almost certainly they are due to decreased availability of free cortisol.

Addressing the second question, our results further suggest that there is no influence of CBG on salivary cortisol responses in men neither in stimulated or in basal levels. The observed range in CBG concentrations is most likely too small to impact on the fraction of available free cortisol. However, high CBG levels were associated with

higher peak levels of total cortisol with identical pre-stress levels. This was in contrast to women, where only the absolute cortisol levels were influenced and no differences in response pattern were observed. ACTH responses to the TSST in men were also associated with CBG levels. In contrast to women, there was a positive association between CBG and ACTH responses. Following the same reasoning as above, this observation might reflect a slight increase in CRF drive to compensate for lower free cortisol levels as would be expected with higher CBG levels. Since the actual differences in CBG and free cortisol levels are much smaller than in women, a down-regulation of CRF receptors might not have occurred.

An alteration of CBG binding capacity has been reported in response to chronic stress (Lynn et al 2003; Marti et al 1997; Tinnikov 1999), and it has also been suggested that CBG does not play a role in the acute stress response. However, there is growing evidence that CBG might play a more dynamic role in regulating glucocorticoid availability to target tissue. Intracellular CBG has been observed in various tissues (see Breuner and Orchinik 2002), including human hypothalamus (Sivukhina et al 2006), which may regulate GC actions by altering local concentrations of cortisol. In addition, the binding of CBG to membrane binding sites leads to activation of intracellular second-messenger cascades. These processes only occur when cortisol is bound to CBG (Nakhla et al 1988; Strel'chyonok and Avvakumov 1991). In a recent study, the role of CBG as a putative dynamic factor regulating the acute stress response was investigated in 9 bird species (Breuner et al 2006). Results indicated that in 5 out of 9 species, binding capacities of CBG reduced significantly, increasing the amount of corticosterone available to tissues. Results from our study, although CBG binding characteristics were not assessed during the time-course of the stress response, do not lend support for a similar mechanism in humans. If CBG binding capacities decreased, the amount of free cortisol, as indicated by salivary cortisol levels, would be much higher in individuals with high CBG levels and high total serum cortisol levels. However, high CBG levels were associated with low salivary cortisol in response to both psychological and physiological stimulation. It is more likely that lower availability of biological active cortisol is compensated at the cellular level by increases in GC sensitivity as observed by Rohleder et al. (2003).

In summary, we investigated a group of OC using women with high CBG concentrations and a group of men with normal CBG levels. High CBG levels, as observed in women, significantly impact on hormonal responses following

pharmacological and psychosocial stimulation. Results further indicate that when CBG levels are low, as observed in men, there seems to be no influence of CBG on the availability of free cortisol. However, an impact of CBG on ACTH and total cortisol responses becomes visible not only in OC using women with exogenously up-regulated CBG levels, but already in men with a much narrower range of CBG levels, possibly reflecting the potential of long-term regulatory actions of CBG on HPA axis characteristics. The results of this descriptive study underscore the importance of CBG as a regulatory element of HPA axis response patterns. For the interpretation of hormonal responses to HPA axis stimulation, CBG has to be taken into account as a potential modifier of hormonal levels.

6.6 References

Bamberger CM, Schulte HM, Chrousos GP (1996): Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 17:245-261.

Breuner CW, Lynn SE, Julian GE, Cornelius JM, Heidinger BJ, Love OP, et al (2006): Plasma-binding globulins and acute stress response. *Horm Metab Res* 38:260-268.

Breuner CW, Orchinik M (2002): Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 175:99-112.

Cole TJ, Harris HJ, Hoong I, Solomon N, Smith R, Krozowski Z, Fullerton MJ (1999): The glucocorticoid receptor is essential for maintaining basal and dexamethasone-induced repression of the murine corticosteroid-binding globulin gene. *Mol Cell Endocrinol* 154:29-36.

de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475.

Dhillo WS, Kong WM, Le Roux CW, Alaghband-Zadeh J, Jones J, Carter G, et al (2002): Cortisol-binding globulin is important in the interpretation of dynamic tests of the hypothalamic--pituitary--adrenal axis. *Eur J Endocrinol* 146:231-235.

Dressendorfer RA, Kirschbaum C, Rohde W, Stahl F, Strasburger CJ (1992): Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement. *J Steroid Biochem Mol Biol* 43:683-692.

Ekins R (1990): Measurement of free hormones in blood. *Endocr Rev* 11:5-46.

Fujimoto VY, Villanueva AL, Hopper B, Moscinski M, Rebar RW (1986): Increased adrenocortical responsiveness to exogenous ACTH in oral contraceptive users. *Adv Contracept* 2:343-353.

Hammond GL (1990): Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocr Rev* 11:65-79.

Kajantie E, Phillips DI (2006): The effects of sex and hormonal status on the physiological response to acute psychosocial stress. *Psychoneuroendocrinology* 31:151-178.

Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH (1999): Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med* 61:154-162.

Kirschbaum C, Pirke KM, Hellhammer DH (1995): Preliminary evidence for reduced cortisol responsivity to psychological stress in women using oral contraceptive medication. *Psychoneuroendocrinology* 20:509-514.

Lynn SE, Breuner CW, Wingfield JC (2003): Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird. *Horm Behav* 43:150-157.

Marti O, Martin M, Gavalda A, Giralt M, Hidalgo J, Hsu BR, et al (1997): Inhibition of corticosteroid-binding globulin caused by a severe stressor is apparently mediated by the adrenal but not by glucocorticoid receptors. *Endocrine* 6:159-164.

Nakhla AM, Khan MS, Rosner W (1988): Induction of adenylate cyclase in a mammary carcinoma cell line by human corticosteroid-binding globulin. *Biochem Biophys Res Commun* 153:1012-1018.

Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH (2003): Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology* 28:916-931.

Rohleder N, Wolf JM, Piel M, Kirschbaum C (2003): Impact of oral contraceptive use on glucocorticoid sensitivity of pro-inflammatory cytokine production after psychosocial stress. *Psychoneuroendocrinology* 28:261-273.

Rosner W (1990): The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocr Rev* 11:80-91.

Siiteri PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, Kuhn RW (1982): The serum transport of steroid hormones. *Recent Prog Horm Res* 38:457-510.

Sivukhina EV, Jirikowski GF, Bernstein HG, Lewis JG, Herbert Z (2006): Expression of corticosteroid-binding protein in the human hypothalamus, co-localization with oxytocin and vasopressin. *Horm Metab Res* 38:253-259.

Strel'chyonok OA, Avvakumov GV (1991): Interaction of human CBG with cell membranes. *J Steroid Biochem Mol Biol* 40:795-803.

Tinnikov AA (1999): Responses of serum corticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. *Endocrine* 11:145-150.

Westphal U (1983): Steroid-protein interaction: from past to present. *J Steroid Biochem* 19:1-15.

Wiegratz I, Jung-Hoffmann C, Kuhl H (1995): Effect of two oral contraceptives containing ethinylestradiol and gestodene or norgestimate upon androgen parameters and serum binding proteins. *Contraception* 51:341-346.

Wiegratz I, Kutschera E, Lee JH, Moore C, Mellinger U, Winkler UH, Kuhl H (2003): Effect of four different oral contraceptives on various sex hormones and serum-binding globulins. *Contraception* 67:25-32.

CHAPTER 7

General Discussion

The aim of the present study was to investigate the influence of common polymorphisms of the GR gene on GR functioning. This study focused on the role of the GR from a psychobiological perspective and scrutinized the association of GR gene variants with HPA axis regulation, with GC sensitivity in three important target tissues and with memory function. Significant associations were revealed for adrenocortical responses to psychosocial stress, with measures of GC sensitivity and with working memory performance. Since results from the experimental work have been discussed separately at the end of each respective chapter, the aim of the general discussion is to integrate these findings and attempt to provide general conclusions. Furthermore, methodological aspects and limitations of the present study will be discussed.

A prominent finding that emerged when results from the different tests are drawn together is that GR gene variants seem to exert their effects in a tissue- and sex-specific way. This is best illustrated by neuroendocrine characteristics and cognitive performance in the 9beta AG and the *Bcl*I GG genotype. In response to psychosocial stress, 9beta AG men displayed the highest levels of ACTH and total cortisol, while women with the same genotype showed similar levels to the other groups. The analysis of GC sensitivity markers further supports sex-specific effects and in addition points towards tissue specificity of the 9beta AG variant. While a relative non-suppression of ACTH and salivary cortisol levels was observed following Dexamethasone administration in men, hormonal levels in women were very similar to the other genotype groups. GC sensitivity of leukocytes and subdermal blood vessels showed no association with the 9beta AG genotype, neither in men nor in women. Examination of working memory performance also revealed a sex-specific effect: 9beta AG women showed the best performance as indicated by lowest reaction times in an item recognition task, while 9beta AG men displayed the highest reaction times. Clear effects, also sex- and tissue-specific, could be revealed for the *Bcl*I GG genotype as well. In men, the *Bcl*I GG genotype was associated with lowest ACTH, total serum and salivary cortisol levels in response to the TSST. Conversely, females of the same genotype showed the highest levels of total serum cortisol in response to stress. GC sensitivity of leukocytes or pituitary was not associated with *Bcl*I GG genotype; interestingly, however, this genotype was related to decreased GC sensitivity of subdermal blood vessels in both men and women. No associations with memory performance were shown for this genotype.

A molecular mechanism providing an explanation for the observed variability in GC responsiveness in general and possibly for the tissue- and sex-specific effects of GR gene variants is discussed in the following paragraphs.

The GR is ubiquitously expressed and has important and diverse roles throughout physiology. Only one GR gene has been identified to this date (Lu and Cidlowski 2005), however, the observed variability in responses to hormone between cells and the diversity of downstream action of activated GR is enormous. This diversity of GC responses can in part be explained by naturally occurring GR isoforms, all produced from a single gene (described in section 2.2.5), but all possessing unique transcriptional regulatory profiles (Lu and Cidlowski 2006). When GR isoforms are co-expressed, transcriptional activity reflects the composite actions of the isoforms, thus providing a mechanism for unique tissue-specific actions on the downstream biology. The way in which cell- and tissue-specific composition of isoforms is achieved is currently unknown, however, it is very likely that different cellular environments can result in differential expression of GR isoforms. One of the factors shown to influence expression of GR isoforms is the presence of the ER22/23EK and the 9beta GR gene variant (see Chapter 4), implying that GR gene variants can influence the fine-tuning of the GC response via effects on GR isoform expression. These effects might be differentially pronounced in different GC responsive cells, giving one potential explanation of the tissue-specific effects of the investigated variants. Apart from effects on isoform generation, another possible explanation for tissue specificity and especially for the observed sex specificity of the investigated polymorphism can be hypothesized. Different cellular contexts might result in varying magnitudes of a GR gene variant's effect on GC signaling. Since the cellular environment in women and men is different with regard to gene expression and hormonal milieu, these differences in signaling environment might account for the differential effects observed (see Chapter 3). It has been demonstrated that gonadal steroids can modulate GR expression (Da Silva 1999; Krishnan et al 2001; Turner 1997). Possibly, penetrance of GR gene variants can be influenced by the cross talk between sex steroid and glucocorticoid pathways.

The following paragraphs are intended to discuss methodological aspects and limitations of this investigation. A general problem with many candidate gene association studies is the failure of consistent replication. Several explanations (Cardon and Palmer 2003; Colhoun et al 2003; Lohmueller et al 2003; van den Oord

and Sullivan 2003; van Rossum et al 2005; see below) have been put forward to explain the observed inconsistencies. In the following, the study design of the present investigation is discussed with regard to critical methodological aspects of genetic polymorphism studies.

a) Function of the genetic variation and prior probabilities of association

The GR is a primary regulator of HPA axis activity; therefore, a good rationale for the association between GR polymorphism and HPA axis regulation exists. The well-documented physiological role of the GR in GC signaling increases the *a priori* justification and probability of an association. A large number of GR gene polymorphism has been described; for the present study, however, only four genetic variants were chosen for investigation. These variants represent, at present state of research, all the functionally relevant polymorphism of the GR. All the investigated variants have been associated before with measures of GC sensitivity, with HPA axis regulation or with phenotypes associated with altered GC signaling (see Chapter 2.2.7). In addition, for three of the investigated polymorphisms, the obtained results from *in vivo* studies have been confirmed with *in vitro* experiments and the molecular basis of the mechanism has been unraveled, further increasing the probability of association.

b) Quality of phenotyping

High quality of phenotyping is essential for obtaining valid results. Classical association studies mostly compare cases with controls, i.e. individuals diagnosed with a disease compared to healthy individuals. Due to complexity and phenotypic heterogeneity inherent in most psychiatric disorders, effect sizes reported are mostly small. It is likely that subtypes of mental disorders with a distinct neurobiological basis exist, implying that the same mental illness can be caused by alterations or deregulations in independent neurotransmitter systems. It is therefore critical to accurately define the phenotype under investigation to increase the likelihood of detecting an association.

Furthermore, instead of using dichotomous variables, study power can be increased by using continuous variables and employing neuroscience based experimental designs. In the present study, we employed valid neuroendocrine challenge paradigms with high sensitivity and specificity. Experiments used allow interpretation of HPA axis functioning on different description levels. On the one hand, *in vivo* and *in vitro* experiments on GC sensitivity provide results specific to the respective target tissue. On the other hand, employing behavioral tests like the TSST provides data on

HPA axis responses in a relevant psychological context. The need to use challenge tests for an appropriate interpretation of a gene variant's effect on HPA axis regulation is illustrated by the findings shown in Figure 7.1. The comparison of genotype groups in a salivary cortisol diurnal profile does not reveal any differences between the groups, whereas marked differences could be shown in response to psychosocial stress (Chapter 3).

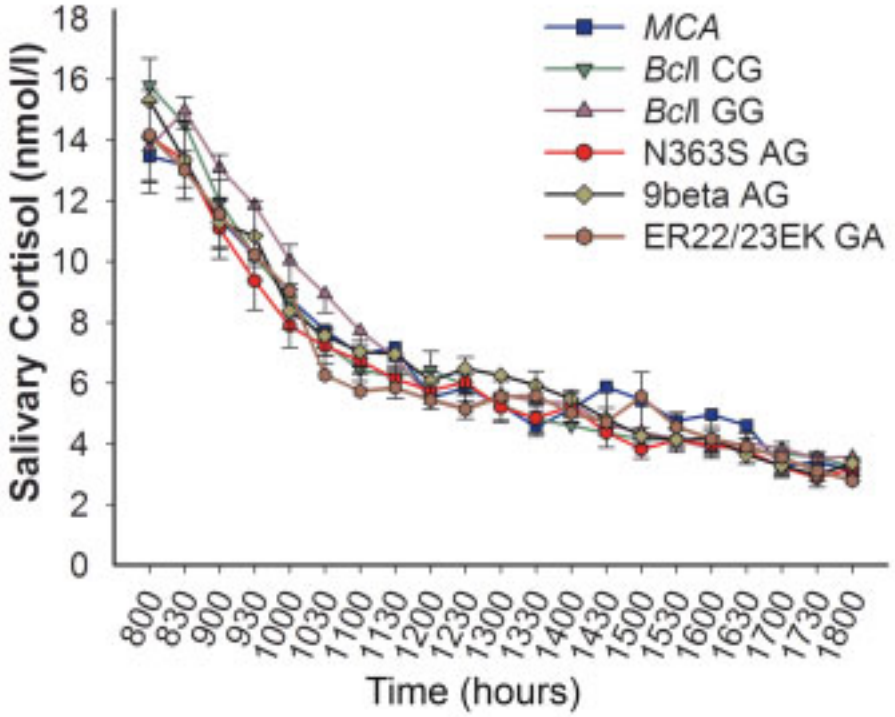

Figure 7.1: *Salivary cortisol levels in a diurnal profile with 30 minute sampling intervals revealing no difference between the genotype groups*

c) Racial heterogeneity and age and sex differences

We sought to ensure high homogeneity of the study population with respect to ethnicity, age and sex. All investigated study participants were of central European descent and we chose to study young adults only, with an age range from 18 to 36 years. Males and females were almost equally distributed and all analyses were conducted controlling for sex effects.

d) Population stratification

Although the genetic background of our participants was not controlled for, population stratification seems unlikely in our study population. The majority of study participants were university students native from various parts of Germany, virtually excluding the possibility of a founder effect.

e) Statistical analysis

False positive results can emerge through multiple testing. The reported p-values in this study were not corrected for multiple testing and therefore have to be interpreted with caution. However, the detailed phenotype assessments might have increased the power sufficiently to identify true positive results. Futhermore, the replication of some of the reported associations, such as the lower HPA axis response to stress observed in the BclI GG genotype in men or the physiological plausibility - inferred from functional in vitro studies - of the reported effects, increase the likelyhood of the results being true positives.

f) Number of individuals studied

Certainly, a limitation of the present study is the small number of individuals studied. Comparing multiple groups, also including variants with low prevalence and using a designs with an extensive phenotyping strategy will inevitably lead to limited sample sizes. Possibly, the strengths of our study indicated above might compensate for the small number of studied individuals and increase power sufficiently to yield valid and interpretable results.

g) Gene - environment interactions

Another limitation is the lack of information about the subjects´ environment. Factors shown to modulate HPA axis regulation are, among others, prenatal factors, postnatal parent-child interactions and socioeconomic status. The effect of these influences might be moderated by an individual's genotype and this issue is further addressed in Chapter 8.

We argue that the GR gene variants under investigation represent all the known relevant SNPs with respect to variability of HPA axis regulation and GC sensitivity in the general population. This view, however, is challenged by two recent investigations employing a haplotype approach, and by a study reporting an association of a SNP located in the regulatory region of the GR with major depression. Rautanen and colleagues (2006) report a common GR haplotype to be associated with basal HPA axis activity and show a modifying role of this haplotype on the association of length at birth with adult phenotypes. The investigated haplotype consists of five markers; one is a synonymous SNP located in exon 9alpha and the other four are intronic. The functional mechanism is unclear, however, expression analyses revealed a reduction of GR mRNA expression level of this

allele. The investigation of haplotypes including multiple polymorphisms has general limitations, since the relative contribution of individual sites of genetic variation cannot be revealed. The haplotype studied by Rautanen et al. does not include any functionally characterized SNPs or any SNPs studied in the present investigation. However, comparison with haplotype data reported by Stevens (2004) allowed inferring presence of a G nucleotide at the *Bcl*I polymorphic site. Possibly, presence of this SNP is responsible, at least in part, for the observed association. Rajeevan et al (2006) found an association of five GR SNPs with chronic fatigue symptomatology and furthermore, haplotype analyses revealed two 5-marker haplotypes associated with the chronic fatigue syndrome. Similar to the study cited above, all SNPs that showed an association were intronic, so the underlying functional mechanism remains elusive for these findings also.

These reports and the replicated associations of the intronic *Bcl*I polymorphism with responses to GC administration and psychosocial stress warrant investigations on the putative functional mechanisms of intronic SNPs. It is possible that intronic SNPs, either alone or in combination, can have an impact on RNA processing or gene transcription by altering recognition sites for RNA splicing factors or transacting enhancers (Cartegni et al 2002). Another explanation that has been put forward is linkage to variants in the promoter region of the gene, potentially having an effect on gene expression. Apart from changes in gene sequences that change the nature of the encoded protein, changes in the amount of protein produced might have equally important consequences (Cox 2004). This amount is determined in part by gene expression, i.e., how much mRNA is transcribed from the relevant DNA sequence. The relative contribution to complex human traits of DNA variants that alter protein structure, versus variants that alter the spatial or temporal pattern of gene expression, is unknown (Hinds et al 2005). There is growing evidence of genetic variations located in the regulatory region of genes possessing functional and behavioral relevance (Caspi et al 2002; Caspi et al 2005; Caspi et al 2003; Hariri et al 2002; Lesch et al 1996). For the GR, the only SNP in the 5´ flanking region that has been associated with HPA axis parameters so far is the so-called *TthIII*1 (rs10052957, Detera-Wadleigh et al 1991), a C/T polymorphic site in the vicinity of exon 1D. Rosmond et al. (Rosmond et al 2000) found increased cortisol levels in a diurnal profile in homozygous carriers of the T allele. Van Rossum et al (2004) investigated the associations of this variant with responses to Dex administration. They found the ER22/23EK G allele in complete linkage with the *TthIII*1 T allele and

carriage of this haplotype was related to relative decreases in GC sensitivity. However, individuals carrying a T at the *TthIII*1 site and the major allele at position 22/23 showed no differences in Dex suppressed cortisol levels, so the authors concluded that the *TthIII*1 T variant seems not to be functional by itself, but only in combination with the ER22/23EK polymorphism. It remains to be investigated if the *TthIII*1 SNP is related to indices of HPA axis activity other than sensitivity to exogenous GC administration. More recently, an association has been reported between a SNP (rs10482605) in the promoter region of the GR and the occurrence of major depression (van West et al 2006). No data on functional studies have been performed with this variant.

GR gene polymorphisms, despite the GR´s importance, most likely account for only a part of the phenotypic variability observed in HPA axis regulation. Variants in other genes are likely to modulate either GR functioning directly or HPA axis regulation via other mechanisms. For instance, a study by Binder et al. (2004) reported an association between a polymorphism in the GR-regulating cochaperone of FKBP5 and increased responses to antidepressant treatment. Recently, DeRijk and colleagues (Derijk et al 2006) investigated a non-synonymous coding SNP in the mineralocorticoid receptor (MR) for association with HPA axis and autonomic responses to the TSST. Results show that carriers of this variant have enhanced responses in cortisol secretion and heart rate to a psychosocial stressor, while functions of aldosterone on sodium balance were unaltered. Furthermore, functional analysis of the MR180V allele revealed a slight decrease in transcriptional activity using cortisol, but not aldosterone, as ligand. These two studies are examples of a hypothesis driven approach of choosing candidate genes involved in HPA axis regulation. Another possibility identifying relevant genes are whole-genome association studies, which will be addressed in Chapter 8.

Following conclusions can be drawn from results of the present study. Common GR gene variants can explain a considerable proportion of response heterogeneity observed in HPA axis responses following psychological stress and sensitivity to exogenous GC administration. Furthermore, the effect of a GR gene variant in one tissue cannot be generalized to others and the effect a GR gene variant has in males cannot be generalized to females.

Results from studies in psychiatric genetics or from investigations on the genetic basis of personality traits, mostly yielding small effects of single variants and reporting difficulties in reproducibility, have been interpreted to imply that psychiatric disease or personality traits must arise from many genes, each having a small effect (Plomin and Crabbe 2000). Results presented in this thesis suggest that variations in a single gene can have significant effects on the functioning of a complex system such as the HPA axis and explain a considerable amount of the observed phenotypic variance. Although limited by small sample size, the experimental nature of the employed design in combination with the high *a priori* probability might have compensated for the discussed limitations. It can be hypothesized that the genetic architecture of a physiological system like the HPA axis, which needs to be reliably activated, executes life-sustaining function and is evolutionary old and well conserved, is less complex than that of higher cognitive functions or personality traits. Therefore, relatively few genes and variations therein can indeed have an impact on characteristics of this neuroendocrine system. Single nucleotide polymorphisms can exert effects on molecular mechanisms on a cellular level and thereby influence the downstream biology of the respective system, leading to alterations in tissues and ultimately affect the entire physiology of an organism. With respect to the GR, it is well possible that minor effects can accumulate over time to finally constitute a risk factor for the development of metabolic, psychiatric or other disease associated with altered GC signaling. In combination with unfavorable environments, such as inadequate diet, low socioeconomic status, or in response to psychological trauma, genetic vulnerability can ultimately lead to manifest pathology. In combination with association studies and future studies on gene-environment interactions, genetic polymorphisms in neuroendocrine or neurotransmitter systems known to be associated with mental disorders might be used as a diagnostic tool for the identification of individuals at risk for the development of psychiatric disorders.

References

Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, et al (2004): Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. *Nat Genet* 36:1319-1325.

Cardon LR, Palmer LJ (2003): Population stratification and spurious allelic association. *Lancet* 361:598-604.

Cartegni L, Chew SL, Krainer AR (2002): Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 3:285-298.

Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW, et al (2002): Role of genotype in the cycle of violence in maltreated children. *Science* 297:851-854.

Caspi A, Moffitt TE, Cannon M, McClay J, Murray R, Harrington H, et al (2005): Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. *Biol Psychiatry* 57:1117-1127.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al (2003): Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301:386-389.

Colhoun HM, McKeigue PM, Davey Smith G (2003): Problems of reporting genetic associations with complex outcomes. *Lancet* 361:865-872.

Cox NJ (2004): Human genetics: an expression of interest. *Nature* 430:733-734.

Da Silva JA (1999): Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* 876:102-117; discussion 117-108.

Derijk RH, Wüst S, Meijer OC, Zennaro MC, Federenko IS, Hellhammer DH, et al (2006): A common polymorphism in the mineralocorticoid receptor modulates stress responsiveness. *J Clin Endocrinol Metab*.

Detera-Wadleigh SD, Encio IJ, Rollins DY, Coffman D, Wiesch D (1991): A TthIII1 polymorphism on the 5' flanking region of the glucocorticoid receptor gene (GRL). *Nucleic Acids Res* 19:1960.

Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, et al (2002): Serotonin transporter genetic variation and the response of the human amygdala. *Science* 297:400-403.

Hinds DA, Stuve LL, Nilsen GB, Halperin E, Eskin E, Ballinger DG, et al (2005): Whole-genome patterns of common DNA variation in three human populations. *Science* 307:1072-1079.

Krishnan AV, Swami S, Feldman D (2001): Estradiol inhibits glucocorticoid receptor expression and induces glucocorticoid resistance in MCF-7 human breast cancer cells. *J Steroid Biochem Mol Biol* 77:29-37.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al (1996): Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 274:1527-1531.

Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN (2003): Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 33:177-182.

Lu NZ, Cidlowski JA (2005): Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell* 18:331-342.

Lu NZ, Cidlowski JA (2006): Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol* 16:301-307.

Plomin R, Crabbe J (2000): DNA. *Psychol Bull* 126:806-828.

Rajeevan MS, Smith AK, Dimulescu I, Unger ER, Vernon SD, Heim C, Reeves WC (2006): Glucocorticoid receptor polymorphisms and haplotypes associated with chronic fatigue syndrome. *Genes Brain Behav*.

Rautanen A, Eriksson JG, Kere J, Andersson S, Osmond C, Tienari P, et al (2006): Associations of body size at birth with late life cortisol concentrations and glucose

tolerance are modified by haplotypes of the glucocorticoid receptor gene. *J Clin Endocrinol Metab*.

Rosmond R, Chagnon YC, Chagnon M, Perusse L, Bouchard C, Bjorntorp P (2000): A polymorphism of the 5'-flanking region of the glucocorticoid receptor gene locus is associated with basal cortisol secretion in men. *Metabolism* 49:1197-1199.

Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE, Donn R (2004): Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. *J Clin Endocrinol Metab* 89:892-897.

Turner BB (1997): Influence of gonadal steroids on brain corticosteroid receptors: a minireview. *Neurochem Res* 22:1375-1385.

van den Oord EJ, Sullivan PF (2003): False discoveries and models for gene discovery. *Trends Genet* 19:537-542.

van Rossum EF, Roks PH, de Jong FH, Brinkmann AO, Pols HA, Koper JW, Lamberts SW (2004): Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms. *Clin Endocrinol (Oxf)* 61:573-581.

van Rossum EF, Russcher H, Lamberts SW (2005): Genetic polymorphisms and multifactorial diseases: facts and fallacies revealed by the glucocorticoid receptor gene. *Trends Endocrinol Metab* 16:445-450.

van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, et al (2006): Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. *Neuropsychopharmacology* 31:620-627.

CHAPTER 8

Outlook

8.1 Whole Genome Associations Studies

Rapid advances in biotechnology make new designs that extend beyond classical candidate gene or linkage studies more feasible. Candidate gene analysis represents a hypothesis driven approach and is a direct test of association between a putatively functional variant, or between a variant assumed to be in linkage with another functional variant, and a phenotypic trait. An alternative approach is indirect association, where a dense map (up to 500,000 SNPs in one assay) of SNPs is tested for association. The advantage of this approach is that is does not require prior determination of functionally important SNPs and might reveal new candidates important for a given phenotype, although the variant had a low prior probability of association inferred from physiology (Carlson et al 2004; Gibbs and Singelton 2006). The application of genome wide association studies has centered on the identification of disease-predisposing alleles; however, whole-genome association studies can also be applied for genetic dissection of polygenic phenotypes. Recently, Papassotiropoulos et al. (2006) used a genome-wide screen to detect SNPs implicated in human episodic memory performance and found a genomic locus encoding the brain protein KIBRA to be associated with memory performance. Investigating individuals stratified into groups according to HPA axis characteristics in response to different stimulation procedures might be a new way of dissecting the genetics of HPA axis phenotypes. Genes that have not been associated with HPA axis characteristics before might be revealed, generating new hypotheses and possibly leading to new ideas and concepts about stress physiology. Furthermore, as genes interact with one another within genetic networks to modulate cellular system and generate specific phenotypes (Bussey et al 2006), specific combinations of polymorphisms might underlie alterations in cellular or physiological characteristics, which can ultimately lead to an effect on the level of system physiology. Using whole genome association is a feasible approach that allows the identification of these specific sets of polymorphisms.

8.2 Imaging Genomics

Imaging genomics is an emerging field in neuroscience integrating genetics with brain imaging techniques (Hariri and Weinberger 2003). The responses of distinct neural circuits during specific visual, auditory, and cognitive or emotional stimuli are assessed, which allows investigating the effects and functional impact of genetic

variants at the level of brain information processing with high sensitivity. Hariri et al. (2002) were the first to apply a functional magnetic resonance imaging (fMRI) paradigm comparing carriers of the „s-allele“ of a common polymorphism of the serotonin transporter (5HTT) with „l-allele“ homozygotes. The physiologic response of the human amygdala was measured during the perceptual processing of fearful and threatening faces and s-allele carriers exhibited a significantly greater amygdala response than l-allele homozygotes. These findings could be replicated and other reports using this approach followed (Canli et al 2006; Furmark et al 2004; Hariri et al 2002).

A study utilizing this method can be conceived for the investigation of GR gene variants´ effect on central information processing. It has been shown that cortisol has a modulatory role on memory processes (see Chapter 5). A potential study design could measure neuronal activity in individuals carrying different GR gene alleles under placebo and under cortisol administration during specific memory tasks. Neuronal activity in the respective regions of interest, e.g. hippocampal structures during declarative memory task or dorsolateral prefrontal cortex during working memory tasks, can be related to memory performance under different treatment conditions.

In addition to functional studies, positron emission tomography (PET) data can yield information about central receptor levels. Research on appropriate tracers for imaging central GRs is well proceeding (Kahn et al 2006), so a protocol for investigating differences in GR density in different GR genotype groups might soon be feasible.

8.3 Gene-Environment Interactions and Epigenetics

In addition to genetic background, stress reactivity in adulthood can be modulated by a number of other factors, including prenatal factors (Seckl and Meaney 2004; Wadhwa 2005), postnatal mother-offspring interaction, as demonstrated in animal research, (Levine 1957; Liu et al 1997; Meaney et al 1988), and early trauma. Epidemiological and case-control studies have investigated the long-term effects of early adverse experiences, such as sexual or physical abuse, emotional neglect or disasters, and it has been shown that these early adversities increase the risk for a great variety of mental disorders and physical illnesses (Kendler et al 2000). In addition, they have been linked to dysregulations in several neurotransmitter and hormonal systems, including the HPA axis (Heim et al 1998; Heim et al 2002). Thus,

evidence suggests that environmental influences can persistently alter HPA axis reactivity, which renders an individual more susceptible to develop stress-related disorders. However, since people show heterogeneity in their response to those causes, a moderating influence of genotype can be assumed. There is a need for studies investigating these gene-environment interactions and furthermore, investigations elucidating the biological mechanisms underlying gene-environment interactions are warranted.

8.3.1 Gene-Environment Interactions

Gene-environment interactions are observed when the effect of exposure to an environmental pathogen on a person's health is conditional on a person's genotype, or conversely, when environmental factors moderate genes' effects on health outcomes (Moffitt et al 2005). Research on gene-environment interactions in psychiatry and psychology has emerged fairly recently. Caspi et al. (2002) reported a moderating effect of monoamin oxidase A (MAOA) genotype on the development of antisocial behavior in maltreated children. Maltreated children whose genotype conferred low expression levels of this neurotransmitter-metabolizing enzyme displayed more often conduct disorder, antisocial personality and adult violent crime compared to children with high-activity MAOA genotype. This report was followed by a study revealing a moderating effect of a common polymorphism in the promoter region of the serotonin transporter (5-HTT) gene on the influence of stressful life events on the development of depression (Caspi et al 2003). Following stressful life events, individuals carrying one or two copies of the 5-HTT s-allele exhibited more depressive symptoms, diagnosable depression and suicidality than individuals homozygous for the l-allele. These results could be replicated (Kendler et al 2005) and other gene-environment findings are emerging (Brookes et al 2006; Caspi et al 2005; Koenen et al 2005; Thapar et al 2005), confirming the validity of this approach. Future studies investigating variants of the GR or other genes involved in HPA axis regulation should include information about early environment. These data must not be limited to early childhood, but should ideally include information about pre- and perinatal factors. Risk factors during pregnancy, such as maternal use of alcohol and smoking, malnutrition and psychosocial stress have been shown to impact on health outcomes in later life. Although very resource intensive, prospective longitudinal studies assessing environmental influences starting at an early stage of pregnancy and retrieving information about critical aspects of development are warranted. This

way, it will be possible to disentangle the relative contributions of environmental factors and to assess the modulating influence of genotype.

8.3.2 Epigenetics

Even after having identified gene-environment interactions, a pivotal question still remains, namely „how an environmental pathogen, especially one that is psycho-social in its nature, gets under the skin to alter the nervous system and generates mental disorders“ (Caspi and Moffitt 2006).

Epigenetic mechanisms can constitute a potential link between early environmental influences and phenotypic outcomes in later life. The term “epigenetic” was originally introduced to describe “The interactions of genes with their environment that bring the phenotype into being “ (Waddington 1942). Currently, it also refers to cellular mechanisms that control gene expression states, independent of changes to the underlying DNA sequence (i.e. DNA methylation, histone and chromatin modification and control of mRNA expression by non-coding RNAs; Jaenisch and Bird 2003; Rakyan and Beck 2006). Interestingly, it has been shown that epigenetic variation exists between monozygotic twins (Fraga et al 2005) and that in some cases these epigenetic variants can be inherited by the offspring (Flanagan et al 2006; Pembrey et al 2006; Suter et al 2004), representing inheritance that is not DNA-based. Most intriguingly, however, it has been demonstrated by Micheal Meaney’s group that environmental influence can change the epigenetic state of a gene. In their work, it was shown that early experience, i.e. variations in maternal care, could affect the development of individual differences in neuroendocrine responses to stress. As adults, the offspring of mothers with high maternal care (more licking and grooming of pups during the first 10 days of life) were less fearful and showed reduced HPA axis responses to stress, increased hippocampal GR mRNA expression, enhanced glucocorticoid feedback sensitivity, as well as changes in several other neurotransmitter systems (Caldji et al 2000; Champagne et al 2001; Francis et al 2000; Francis et al 2002; Liu et al 2000a; Liu et al 2000b; Meaney et al 2002). Cross-fostering studies revealed that most of these effects are non-genomically transmitted from one generation to the other. Subsequent studies focused on the biological mechanisms underlying the sustained alterations in gene expression in the offspring as a consequence of variations in maternal care. Interestingly, it was shown that variations in maternal care led to stable alterations of DNA methylation patterns. Low maternal care results in increased methylation of the nerve growth factor-inducible

protein A (NGFI-A) binding site located in the GR gene exon 1_7 promoter, leading to decreased GR expression. Remarkably, expression of exon 1_7 containing mRNA seems to be unique to hippocampus (McCormick et al 2000). Altered expression of GR mRNA by methylation of exon 1_7 can therefore reduce GR protein levels specifically in hippocampal sites. Since negative feedback on HPA axis activity is substantially mediated by hippocampal GR, differential methylation underlies the neuroendocrine alterations observed in offspring of low caring mothers. These findings define a basis for the development of stable differences in endocrine function and behavior over the life span and provide evidence for a biological substrate, namely, the epigenetic state of a gene (Weaver et al 2004; Weaver et al 2002).
The question remains, if a similar mechanism also exists in humans. A first step in transferring these findings to humans was the thorough characterization of the 5` untranslated region (UTR) of the human GR gene by Turner and Muller (2005). At least eleven splice variants were revealed and these are based on seven exon 1s, four of which were so far unknown. As previously reported for the rat, alternative first exons are located within a CpG rich island, a region with high rat – human sequence identity score. This 3-kbp CG-rich region contains 7 alternative rat exons 1s, and their six homologous human splice variants. The human GR exon 1s and their respective promoters show remarkable similarity with their rat orthologues.
Investigation of the methylation pattern of exon 1F promoter, the human orthologue to rat exon 1_7, revealed no differential methylation in human hippocampus (Moser et al., in prep). In contrast to the findings in rats, none of the two CpG motifs within the NGFI-A binding site of the human hippocampus GR exon 1F promoter was methylated. Weaver et al. (2004) reported methylation of two CpG dinucleotides in the NGFI-A binding site, where the 5' CpG displayed differences in methylation dependent on maternal care, whereas the 3' CpG site was constantly methylated, independent of maternal care. Examination of human hippocampi found both CpGs in the NGFI-A binding site unmethylated, possibly indicating general differences in promoter methylation patterns in humans and rats.
Despite the homology in genomic organization, there is a crucial difference between rat and humans with regard to tissue distribution. While exon 1_7 in the rat is central nervous system specific, the human orthologue exon 1F is not, but rather exon 1D, which is expressed in hippocampus only. Based on the tissue specific expression of alternative exon 1s in humans, it can be hypothesized that exon 1D promoter is actually the target for differential methylation dependent on environmental variation.

This question can be addressed by investigation of the promoter region of the hippocampus specific exon 1D. Analysis of this genomic region ought to include the exploration of putative transcription factor binding sites and the determination of the corresponding methylation pattern. *In silico* analysis revealed 26 CpG dinucleotides in the proximal promoter region of the GR alternative exon 1D, representing potential targets for differential promoter methylation. Furthermore, several transcription factor binding sites were identified using BIMAS Promoter Scan Software (http://bimas.dcrt.nih.gov/molbio/proscan) and Transfac (http://www.gene-regulation.com/pub/databases.html), including SP1, ELK-1, YY1 and NFMUE1. Additionally, a DNA sequence showing high homology to a NGFI-A binding site has been identified. This sequence GCGGCGGGCG (located at position -99 to -89 in relation to transcription initiation site of exon 1D) is highly similar to several probes used to identify NGFI-A DNA-binding specificity (Swirnoff and Milbrandt 1995). Furthermore, the putative binding site is located in a highly conserved genomic region (comparison of five different species; Turner, personal communication).
The discovery of putative binding sites for transcription factors in the GR exon 1D promoter region and interindividual differences in methylation pattern in human hippocampus could potentially reveal the basis for an epigenetic mechanisms linking behavioral and environmental variation to molecular changes in humans, underlying neuroendocrine alterations and increased susceptibility for psychopathology.

8.4 References

Brookes KJ, Mill J, Guindalini C, Curran S, Xu X, Knight J, et al (2006): A common haplotype of the dopamine transporter gene associated with attention-deficit/hyperactivity disorder and interacting with maternal use of alcohol during pregnancy. *Arch Gen Psychiatry* 63:74-81.

Bussey H, Andrews B, Boone C (2006): From worm genetic networks to complex human diseases. *Nat Genet* 38:862-863.

Caldji C, Francis D, Sharma S, Plotsky PM, Meaney MJ (2000): The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology* 22:219-229.

Canli T, Qiu M, Omura K, Congdon E, Haas BW, Amin Z, et al (2006): Neural correlates of epigenesis. *Proc Natl Acad Sci U S A* 103:16033-16038.

Carlson CS, Eberle MA, Kruglyak L, Nickerson DA (2004): Mapping complex disease loci in whole-genome association studies. *Nature* 429:446-452.

Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW, et al (2002): Role of genotype in the cycle of violence in maltreated children. *Science* 297:851-854.

Caspi A, Moffitt TE (2006): Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci* 7:583-590.

Caspi A, Moffitt TE, Cannon M, McClay J, Murray R, Harrington H, et al (2005): Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. *Biol Psychiatry* 57:1117-1127.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al (2003): Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 301:386-389.

Champagne F, Diorio J, Sharma S, Meaney MJ (2001): Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc Natl Acad Sci U S A* 98:12736-12741.

Flanagan JM, Popendikyte V, Pozdniakovaite N, Sobolev M, Assadzadeh A, Schumacher A, et al (2006): Intra- and interindividual epigenetic variation in human germ cells. *Am J Hum Genet* 79:67-84.

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, et al (2005): Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 102:10604-10609.

Francis DD, Champagne FC, Meaney MJ (2000): Variations in maternal behaviour are associated with differences in oxytocin receptor levels in the rat. *J Neuroendocrinol* 12:1145-1148.

Francis DD, Young LJ, Meaney MJ, Insel TR (2002): Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. *J Neuroendocrinol* 14:349-353.

Furmark T, Tillfors M, Garpenstrand H, Marteinsdottir I, Langstrom B, Oreland L, Fredrikson M (2004): Serotonin transporter polymorphism related to amygdala excitability and symptom severity in patients with social phobia. *Neurosci Lett* 362:189-192.

Gibbs JR, Singleton A (2006): Application of Genome-Wide Single Nucleotide Polymorphism Typing: Simple Association and Beyond. *PLoS Genet* 2.

Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, et al (2002): Serotonin transporter genetic variation and the response of the human amygdala. *Science* 297:400-403.

Hariri AR, Weinberger DR (2003): Imaging genomics. *Br Med Bull* 65:259-270.

Heim C, Graham YP, Heit SC, Bonsall R, Miller AH, Nemeroff CB (1998): Increased sensitivity of the hypothalamic-pituitary-adrenal axis to psychosocial stress in adult survivors of childhood abuse. *Soc Neurosci Abst* 28:201.212.

Heim C, Newport DJ, Wagner D, Wilcox MM, Miller AH, Nemeroff CB (2002): The role of early adverse experience and adulthood stress in the prediction of neuroendocrine stress reactivity in women: a multiple regression analysis. *Depress Anxiety* 15:117-125.
Jaenisch R, Bird A (2003): Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 33 Suppl:245-254.
Kahn MG, Konde E, Dossou F, Labaree DC, Hochberg RB, Hoyte RM (2006): Microwave-enhanced nucleophilic fluorination in the synthesis of fluoropyridyl derivatives of [3,2-c]pyrazolo-corticosteroids, potential glucocorticoid receptor-mediated imaging agents. *Bioorg Med Chem Lett* 16:3454-3458.
Kendler KS, Bulik CM, Silberg J, Hettema JM, Myers J, Prescott CA (2000): Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. *Arch Gen Psychiatry* 57:953-959.
Kendler KS, Kuhn JW, Vittum J, Prescott CA, Riley B (2005): The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication. *Arch Gen Psychiatry* 62:529-535.
Koenen KC, Saxe G, Purcell S, Smoller JW, Bartholomew D, Miller A, et al (2005): Polymorphisms in FKBP5 are associated with peritraumatic dissociation in medically injured children. *Mol Psychiatry* 10:1058-1059.
Levine S (1957): Infantile experience and resistance to physiological stress. *Science* 126:405.
Liu D, Caldji C, Sharma S, Plotsky PM, Meaney MJ (2000a): Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. *J Neuroendocrinol* 12:5-12.
Liu D, Diorio J, Day JC, Francis DD, Meaney MJ (2000b): Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci* 3:799-806.
Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, et al (1997): Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 277:1659-1662.
McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, et al (2000): 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol Endocrinol* 14:506-517.
Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM (1988): Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 239:766-768.
Meaney MJ, Brake W, Gratton A (2002): Environmental regulation of the development of mesolimbic dopamine systems: a neurobiological mechanism for vulnerability to drug abuse? *Psychoneuroendocrinology* 27:127-138.
Moffitt TE, Caspi A, Rutter M (2005): Strategy for investigating interactions between measured genes and measured environments. *Arch Gen Psychiatry* 62:473-481.
Papassotiropoulos A, Stephan DA, Huentelman MJ, Hoerndli FJ, Craig DW, Pearson JV, et al (2006): Common kibra alleles are associated with human memory performance. *Science* 314:475-478.
Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, Golding J (2006): Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* 14:159-166.
Rakyan VK, Beck S (2006): Epigenetic variation and inheritance in mammals. *Curr Opin Genet Dev*.
Seckl JR, Meaney MJ (2004): Glucocorticoid programming. *Ann N Y Acad Sci* 1032:63-84.
Suter CM, Martin DI, Ward RL (2004): Germline epimutation of MLH1 in individuals with multiple cancers. *Nat Genet* 36:497-501.
Swirnoff AH, Milbrandt J (1995): DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol Cell Biol* 15:2275-2287.
Thapar A, Langley K, Fowler T, Rice F, Turic D, Whittinger N, et al (2005): Catechol O-methyltransferase gene variant and birth weight predict early-onset antisocial

behavior in children with attention-deficit/hyperactivity disorder. *Arch Gen Psychiatry* 62:1275-1278.
Waddington C (1942): The epigenotype. *Endeavour* 1:18-20.
Wadhwa PD (2005): Psychoneuroendocrine processes in human pregnancy influence fetal development and health. *Psychoneuroendocrinology* 30:724-743.
Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al (2004): Epigenetic programming by maternal behavior. *Nat Neurosci* 7:847-854.
Weaver IC, Szyf M, Meaney MJ (2002): From maternal care to gene expression: DNA methylation and the maternal programming of stress responses. *Endocr Res* 28:699.