Fachbereich I – Psychobiologie

Involvement of Mineralocorticoid Receptor Polymorphisms in Human Cognitive Function

Dissertation

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Für meine Eltern

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Danksagung

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Abbreviations

A	adenine
AA	approach-avoidnace
ACC	anterior cingulate cortex
ACTH	adrenocorticotropic hormone
ADX	adrenalecomized
AF-1	activation function I
AG	amygdala
ANOVA	Analysis of Variance
ANT	*Attention Network Task*
BIS	behavioral inhibition scale
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
bp	base pair
C	cytosine
CA	cornu ammonis
CaMKII	calcium/calmodulin dependent protein kinase type II
CAR	cortisol awakening response
CB-1	cannabinoid receptor type I
CBG	cortisol binding globulin
CeA	central amygdala
CORT	corticosterone
COMT	catechol-O-methyltransferase
CRH	corticotrophin releasing hormone
DBD	DNA binding domain
DEX	dexamethasone
DNA	deoxyribonucleic acid
DRD4	dopamine receptor D4
E	epinephrine
EEG	electroencephalogramm
EOG	electrooculogram
ERP	event related potential
fMRI	functional magnet resonance imaging
G	guanine
GAS	*General Adaptation Syndrom*
GABA	gamma amino butric acid
GC	glucocorticoid
GR	glucocorticoid receptor
GWAS	genome wide association studies
HC	hippocampus
HPA axis	hypothalamus-pituitary-adrenal axis
HWE	Hardy Weinberg equilibrium
i.c.v.	intracerebroventricular
IL-6	interleukin 6
ITI	inter trial interval
kb	kilobase
kDa	kilo Dalton

Abbreviations

LBD	ligand binding domain
LC	locus ceruleus
LTD	long term depression
LTP	long term potentiation
MAOA	monoaminoxidase A
MDR PGP	multidrug resistance P glucoprotein
MeA	medial amygdala
mEPSC	mini excitatory post synaptic current
mIPSC	mini inhibitory post synaptic current
MIST	*Montreal Imaging Stress Test*
MR (h)	mineralocorticoid receptor (human)
MRI	magnet resonance imaging
mRNA	messenger ribonucleic acid
ms	millisecond
MWM	*Morris Water Maze*
µV	microvolt
NAc	nucleus accumbens
NE	norepinephrine
NLS	nuclear location signal
NR3C1	nuclear receptor subfamily 3, gloup C, member 1
NR3C2	nuclear receptor subfamily 3, group C, member 2
NTD	N-terminal domain
NTS	nucleus tractus solitarius
OC	oral contraceptives
PFC (l, m, vm)	prefrontal cortex (lateral, medial, ventromedial)
PHA-1	pseudohypoaldosteronism type 1
POMC	proopiomelanocortin
PTSD	post traumatic stress disorder
PVN	paraventricular nucleus
RT	reaction time
SAGE	serial analysis of gene expression
SD	standard deviation
SCN	suprachiasmatic nucleus
SNP	single nucleotide polymorphism
SPECT	single photon emission computed tomography
SRC	steroid receptor coactivator
T	thymine
TPH2	tryptophan hydroxylase 2
TSST	*Trier Social Stress Test*
VTA	ventral tegmental area
WCST	*Wisconsin Card Sorting Test*
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine (serotonin) transporter
11βHSD2	11-β steroid dehydrogenase type 2

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CHAPTER 1

Introduction and Outline

1.1 Introduction

"Stress is a dangerous and useless word. It may seem useful because it is a unifying word, but it unifies our ignorance rather than our knowledge."
Zanchetti (1972)

It might still hold true that the term of 'stress' is a unifying word and subsummarizes multiple physical and psychological processes. But the progress that has been made in the field of stress research might anyhow contribute to the falsification of this citation and the aim of the the present thesis is to unify the knowledge that has been gained since.

The link between 'Stress' and physical illness as well as psychological well-being has long been made and growing interest of mechanisms mediating this association led to extensive investigation of the body and mind phenomenon of stress. Early observations in the clinical field concentrated rather on the physical outcome of stress like colitis, ulcers or, as a prominent example, coronary disease. Vulnerability factors were likely to be described as personality traits or types, emphasizing the idea of inheritance of vulnerability on one side but also taking into account environmental factors as education, occupation or social class. Also, the idea that personality traits and thereby the predisposition to develop related diseases can be inherited was integrated in these psychosomatic models (e.g. Stewart 1950). In the same time but coming from a completely different direction the term of 'stress' was investigated focusing on the biological impact of 'stressful' challenges on an organism, avoiding a negative connotation of the term (Cannon 1932; Selye 1956). In these stress theories, stress is seen as an adaptive behavior enabling the body to face and survive physiological or psychological challenges. Nevertheless, enduring stressful conditions can promote or sustain disease processes if adequate coping is not possible and if the organism is faced with chronically adverse situations. To date, the relation between stressful live events and mental disorders has well been established. In the etiology of virtually all psychiatric disorders stress, or – more precisely cortisol – plays a crucial role (Young 2004). In humans, cortisol is the end product of an adaptive and stress responsive biological system: the hypothalamus-pituitary adrenal (HPA) axis, a hormonal system that is hierarchically organized and mediates the endocrine reaction to a physical or psychological challenge. About hundred years of stress research now have led to an enormous knowledge about the characteristics of a challenging environment, psychological coping mechanisms like health related behaviors and cognitive styles, lifetime phases of enhanced vulnerability to stressors or critical life events and other influencing factors. Above all, the comprehension of physiological processes elicited by stress has progressed a lot, now integrating the description of very specific molecular pathways and interdependences of different stress related systems. Focusing on

HPA axis function, another field has become of big interest, namely the description, localization and modulation of stress related cognitions. Cortisol itself in humans and other glucocorticoids (GCs) in other species have been found to alter cognitive processes such as learning and memory, and interindividual differing characteristics of the HPA axis nowadays are known to influence stress related cognitions (LeBlanc 2009). Therein, brain structures mediating those processes can be characterized by co-expression of two receptors that bind to cortisol in central structures and that mediate the tight self-regulating feedback of HPA axis function: the high affinity mineralocorticoid receptor (MR) scrutinized for its influences on cognitive functions in this thesis and the ten fold lower affine glucocorticoid receptor (GR). Interesting for stress related cognitions and moreover influencing MR/GR mediated HPA axis feedback are, above all, three brain areas that are implicated in practically all kinds of cognition: the hippocampus, amygdala, and the prefrontal cortex. Their influence on GC secretion differs between subregions, follows distinct time courses and is known to be stressor specific. A dynamic interplay of the two receptors affecting the excitability of neurons in the stress network of the brain at different levels of circulating GCs adds further variables that have to be taken in mind when assessing the influence of possible stressors on the individual stress response. Imbalance of MR:GR mediated actions are influenced by a variety of molecular changes in activation of the receptors, their transport and/or localization and clearly the transcription of the coding genes that can be described as molecular susceptibility pathways (de Kloet et al. 2009). Altogether the HPA axis is a fine tuned, continuously working system that can easily be altered in function due to multiple fast acting, self referencing, and interrelated circuits. So, the selective pharmacological blockade of MR to investigate its functions in human cognition might disturb this equilibrium. But how can one assess the function of a brain specific receptor for GCs if not by selective blockade? And how can one create conditions of differing variance in a system that only has a narrow range of exclusive functioning – because with elevating levels of circulating GCs GR comes into play?

An important step in stress research therefore has been the development of methods that are capable to provide images of the working brain and the implementation of laboratory psychosocial stress tasks. Knowledge has grown concerning biographic, socioeconomic, psychological and health related variables that, in consequence, can be controlled for in future experiments. The other side contributing to individual predispositions to detect, interpret and cope with stressful situations is the genetic background of the organism. In rodent models, the improvement of genetic modification techniques have yet lead to new insights of involvement of a variety of genes and their function in stress. In human research, the deciphering of the whole genome sequence in 2004 (International Human Genome Sequencing Consortium 2004) has revolutionized the field of medical and behavioral genetic

research. Insights in the exact sequence of the human genome and identification of the structure of all known approximately 19,000 genes and more importantly, the information about differences in the genetic make-up of individuals have had a huge impact on what we know today. The most common type of gene variants that differ between individuals is the change of one base pair to another and is thus called a single nucleotide polymorphism (SNP). Per definition evolutionary meaningful SNPs occur in at least 1% of the population and, as work still is progressing, their total number in the last few years has increased from 10 million to around 30 million SNPs in the human genome (http://www.ncbi.nlm.nih.gov/sites/entrez). The location and structure of the MR gene or the gene for nuclear receptor subfamily 3, group C, member 2 (NR3C2) has been discovered in 1987 (Arriza et al. 1987) but only the information about individual differences in its sequence can give deeper insights in its association with stress related cognitions and on proneness or vulnerability to psychiatric illness.

A considerable number of SNPs in genes that code for 'players' in stress regulation has been described for their influence on HPA axis outcome and their role in stress related disease (Derijk et al. 2008; Gillespie et al. 2009) but the question of GC receptor function in human cognitive function still remains to be precisely described.

In order to disentangle the interplay between brain structures, possibly being targets of HPA axis dysfunctions and in the purpose to develop suitable endophenotypes of mental diseases further studies of genetic variants and their impact on distinct cognitive processes is needed.

In this thesis experimental work will be presented intending to characterize cognitive functions affected by MR polymorphisms. The aim is to assess such by behavioral testing in distinct cognitive paradigms and to relate those to physiological measures of brain activation as well as to characterize a relation of MR with cortisol secretion. By this, the use of naturally occurring genetic variation as an example for differing biological functioning of the resulting protein seems promising as an approach to shed light to the involvement of MR in human cognitive function.

1.2 Outline

The investigation of associations between temperament, personality factors or cognitive styles on the one hand and physical or psychiatric illness has a long-standing tradition in which for example the treatment method of cognitive behavioral psychotherapy is rooted. This method, in turn, has not only been used to ameliorate symptoms of psychiatric diseases but also finds implementations in somatoform disorders (Witthoft and Hiller 2010) respectively is applied in the case of co-morbid depression in cardiovascular disease (Summers et al. 2010). As underlying mechanism influencing virtually all psychiatric and a wide range of physical diseases the HPA axis has come into play. Many factors affect both,

psychiatric disease and HPA axis regulation, to name only some, there are sex, and health related behaviors like smoking or exercise and socioeconomic status. To account for an individual's risk to suffer from a high long-term allostatic load, the genetic disposition is an important mediator, potentially rendering an organism susceptible to distinct environmental influences and/or protecting it from harmful consequences of other factors. Integrating genetic methods in bio-psycho-medical research yet have led to new insights in molecular mechanisms of cognition, behavior and health outcomes but suffer from methodological problems because of which results are difficult to replicate (Burmeister et al. 2008). In the experimental work presented in this thesis, we chose candidate gene approach to phenotype behavioral aspects, differentially mediated by two MR polymorphisms that have been shown to be functional for the transcriptional activity of ligand bound MR. The aim of this investigation is to characterize the functioning of MR variants (-2G/C, I180V) in attention networks and set shifting and further screen for an impact of those variants in HPA axis activity and reactivity.

The rational bases of the research strategy for the investigation presented in this thesis will be reflected in the general introduction of the present **Chapter 1**. In **Chapter 2** the theoretical background on the topic is presented, giving a brief introduction in the biopsychological idea of stress and the endocrine system mediating the beneficial and adverse outcomes of stress. In the following the focus will be on cerebral structures that are under the influence of the two dominant GC signaling receptors therein emphasizing behavioral aspects affected by stress. Functions of the MR protein and the genetic structure will be described in detail, concentrating on central, GC related processes as opposed to its meaning in salt-water homeostasis. **Chapter 3** outlines aspects of behavioral measures (reaction times and error rates) for two cognitive tests referring to the different MR genotype groups. In **Chapter 4** electrophysiological outcomes of brain activation, assessed by the method of electroencephalograms (EEG) are presented for the different MR genotype groups. Cortisol data in respect to genetic group differences is presented in **Chapter 5** in form of salivary free cortisol measures for daytime cortisol secretion and cortisol release during the test session. **Chapter 6** provides a general discussion of the presented findings, followed by an outlook delineating future research directions in **Chapter 7**.

Chapters 3 to 5 are written so that they can be read separately and may therefore partly contain unavoidable redundant information. Not all of the subjects taking part in the main study could be integrated in all analyses what leads to slight variations in numbers of participants in the different chapters. Experimental work that is presented in this thesis was conducted in cooperation with PD Dr. Stefan Wüst, Prof. Dr. Hellhammer and Dr. Ewald Naumann from the University of Trier and with Dr. Roel de Rijk from the University of Leiden and the Leiden/Amsterdam Center for Drug Research (LACDR).

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CHAPTER 2

14

Theoretical Background

2.1 Stress – a biopsychological phenomenon

2.1.1 Stress Definition

In everyday's life, the word 'stress' is frequently used and – in most of the cases – it means that somebody is facing a subjectively challenging situation. Normally standing for the impact of tension or pressure on material, the term 'stress' has early been used to describe the impact of psychological and physical demands on an organism adapting on acutely or chronically changing environmental factors.

The first one using the concept of stress as a psychobiological reaction was Walter Cannon who described the "fight-or-flight" reaction of an organism to a stressful event. This first definition was based on the biological reaction of secreted catecholamines, most notably epinephrine and nor-epinephrine and their actions on the sympathetic nervous system. It is characterized by enhanced central and cardiac perfusion and cardiac activity, constriction of peripheral vasculature and increased blood flow in muscles as well as decreased intestinal activity (Cannon 1914; Cannon 1915; Cannon 1932). Consequences of chronic stress were integrated in a model by Hans Selye (1936), who formulated the theory of the General Adaptation Syndrom (GAS). Symptoms consist of enlargement of the adrenal gland; atrophy of the thymus, spleen and other lymphoid tissue and gastric ulcerations and develop in three stages: alarm (acute stress reaction including cortisol secretion), resistance (hyperactive and -reactive system) and exhaustion (long term consequences of chronic stress) (Selye 1980; Selye 1998). The long term consequences of stressful life circumstances have shown to be associated with a wide variety of disorders or clinically relevant symptoms ranging from physical symptoms (cardiovascular problems, diabetes, intestinal dysfunctions…) to psychological symptomatology like depression (Pariante et al. 1995; Holsboer 2000), anxiety (Abelson and Curtis 1996) or post traumatic stress disorder (Yehuda 1997). Despite mechanisms mediating the effect between dysregulated endocrine stress systems and psychiatric diseases remain to be unraveled, hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis (see below) seems to be a risk factor in the development of bipolar disorder or schizophrenia (Rybakowski and Twardowska 1999; Ryan et al. 2004; Daban et al. 2005; see Pariante 2008 for a review).

Hans Selye was the first to attract notice to the term 'stress' in the field of biomedical research. His definition of stress emphasized the biological characteristics of the autonomic, endocrine and immune response to unspecific stimuli that he only characterized by their importance for the following reaction. Therefore, Selye's stress definition can be seen as reaction based model of the phenomenon of stress. Other definitions attempted to include the meaningfulness of potentially stressful stimuli and focused on the interaction between the perceiving individual and the stressful stimulus. Lazarus and Folkman pointed out that the level of stress depends on the evaluation of internal resources of the perceiving individual to

cope with the given situation. In this tradition stress can be seen as a dynamic, multilevel process including the interaction of the stress stimulus (input), the processing system and the stress response (output) (Levine 1991). Stress is not always necessarily harmful. Normally it enables the organism to face a potentially harmful situation, to provide resources to resolve this situation and to enable recovery after cessation of the stressor. Hans Selye formulated in 1956, *"stress is not necessarily something bad - it all depends on how you take it. The stress of exhilarating, creative successful work is beneficial, while that of failure, humiliation or infection is detrimental."* (Selye 1956). Therefore, a widely accepted definition of stress in today's psychobiological research pronounces the importance of homoeostasis of a system that constantly adapts to environmental challenges (Chrousos and Gold 1992). Integrating factors like genetic background, lifestyle habits or developmental and life experiences, the concept of 'allostatic load' has been developed to circumscribe the 'wear and tear' on the whole organism (literally meaning "maintaining stability, or homeostasis, through change"). The brain is the key target for allostatic load and for restoring 'allostasis' – the adaption to challenging conditions. It is the organ that decides what is stressful or threatening and that determines the behavioral and physiological response. Four types of allostatic load have been characterized by the activity and reactivity different physiologic systems, including cortisol secretion. There can either be frequent occurrence of stressors, the incapacity to habituate on such, the inability to shut down a stress response or inadequate responses that may lead to compensatory hyperactivity of other systems. In everyday's life of humans, stressors that elicit a typical 'fight or flight' response will be the exception and long term impact of frequent or chronic 'low level' stress that leads to adaptive behavior whether health promoting or damaging are more probable to lead to physiological, cognitive and behavioral long term adaption (Sterling and Eyer 1988; McEwen 2000; McEwen 2006).

2.1.2 The psychobiological stress response

Psychobiological stress research in humans has one major problem: most of the studies on beneficial and deleterious effects of stress are carried out in rodents and other animals and comprise a well controllable environment, specific acute and chronic stress paradigms (that underlie different ethic guidelines than in humans) and the possibility to control those over time. In humans, it had already turned out to be difficult to elicit an acute stress reaction that works generally and not only for a low percentage of subjects. A 'normal' stress reaction still is likely to be described as the required reaction of a non-human vertebrate facing a predator and being forced to 'fight or flight' (Cannon 1915; Sapolsky et al. 2000).

The acute stress response is governed by two, by positive feedback loops interconnected systems: the locus ceruleus (LC)-norepinephrine (NE) sympathetic system, located in the brain stem and the centrally widespread corticotrophin releasing hormone (CRH) system.

Activation of the LC-NE system triggers the secretion of NE from a dense neuronal network throughout the brain and leads to increased vigilance, arousal and anxiety within seconds. The CRH system on the other hand activates the HPA axis and so is followed by the release of from paraventricular nucleus (PVN) of the hypothalamus in the portal blood system which subsequently leads to the release of adrenocorticotropic hormone (ACTH) to the periphery. Within about ten minutes, the adrenal cortex secretes glucocorticoids (GCs). In the periphery, GCs influence multiple processes of energy mobilization, cerebral glucose transport and consumption, have complex acute effects on immune processes or, at very high levels can induce suppression of reproductive functions (Sapolsky et al. 2000). Physiological challenge as pain, hemorrhage or humoral homeostatic (i.e. glucose/insulin level changes) or inflammatory signals elicit comparable HPA axis responses like psychological stressors. Therefore it has been proposed to assume two modes of the HPA axis, one being reactive to physical stress, eliciting a fast and reflexive HPA response by nociceptive or humoral sensory pathways or visceral afferents via structures that directly innervate the PVN. The second, rather psychological one is triggered by centrally governed detection or expectance of stressors in form of classically or contextually conditioned stimuli (Herman et al. 2003). In animal research examples for such a stressor would be conditioned fear responses or social stress paradigms whereas in humans psychosocial stress can be provoked by laboratory stressors or can be investigated in assessing different sources of HPA axis activity or reactivity in daily life. Laboratory stressors that reliably can provoke an HPA axis response were found to include the psychological characteristics 'ego involvement/motivational performance', 'uncontrollability' and 'social evaluative threat' (Mason 1968; Dickerson and Kemeny 2004). Another possibility to investigate the consequences of stress on HPA axis function in humans is the assessment of real life stressors in the line of the allostatic load concept and would comprise early adverse experiences (even prenatal), traumatic events, job stress (or other daily hassles) as well as health related behaviors or the assessment of dispositional variables like personality factors or the individual's genetic background.

2.2 HPA axis activity and reactivity: homeostatic interplay of two glucocorticoid receptors

2.2.1 GC negative feedback: the role of glucocorticoid receptor

An acute stress reaction of the HPA axis is terminated by efficient negative feedback which is modulated on multiple levels. Rapid autoregulatory and interacting inhibitory mechanisms have been described for NE and CRH (Chrousos 2007). As the effector hormone of the HPA axis GCs exerts its function in the restoration of homeostasis mainly by the glucocorticoid receptor (GR) in (almost) all central and peripheral tissues (Munck et al. 1984; Chrousos et

al. 1993). Bound to its ligand, the GR acts as transcription factor and transactivates or represses the expression of numerous genes that are involved in GC mediated long-term changes. In the brain, GR is abundantly expressed in neurons of the PVN and in the anterior pituitary and for example seems to be involved in the regulation of the gene coding for proopiomelanocortin (POMC) (Drouin et al. 1993). Further targets of GC negative feedback are the hippocampus (HC) and medial prefrontal cortex (mPFC). Inhibition of GC secretion by these structures has been shown to be stressor specific and so HC, AG and PFC primarily react to stressors that include psychological components and are not due to purely physical challenge (Herman et al. 2003). In humans, the meaning of GR negative feedback on HPA axis activity has been proven by pharmacological blockade , genetic association studies (Wüst et al. 2004) or studies in clinical populations with altered HPA axis function (i.e. Spijker and van Rossum 2009 for a review).

2.2.2 The mineralocorticoid receptor in HPA axis function – tonic inhibition or proactive role?

The ubiquitous expression of GR indicates that stress is a whole body and brain phenomenon and the receptor's role has been formulated as to prevent tissue damage through chronically enhanced GCs. But besides from protecting the organism against high stress, another important role of the HPA axis is to regulate the diurnal rhythm of GC secretion that in humans is characterized by rising cortisol levels during the second half of the night, a morning peak and declining levels over the course of the day. Distinct from rising cortisol levels in the morning, an additional increase of cortisol has been observed as a reaction to awakening. The cortisol awakening response (CAR) arises directly and peaks about 30 minutes after morning awakening (Born et al. 1999; see Clow et al. 2004 for a review).

Under normal conditions, hypothalamic drive to ACTH and GC secretion underlies the circadian drive, mediated by efferent connections from the CLOCK system in the suprachiasmatic nucleus (SCN) that project to CRH/AVP containing neurons on the PVN (Nader et al. 2010) and any activity can be considered as consequence of constitutive corticosteroid secretion (Akana et al. 1992; Dallman et al. 1994). In the periphery, GCs are bound to a wide extend (ca. 95%) on cortisol binding globulin (CBG) and albumin which hamper the dislocation of these small lipophilic peptides into the brain. The resting 5% of unbound GCs binds to two receptors with different affinity: the GR and the mineralocorticoid receptor (MR), which has a tenfold higher affinity for corticosteroids then the GR. At minimal plasma corticosterone (CORT) daytime levels, in rats the MR (or type 1 receptor) is occupied to 80% compared to GR (or type 2 receptor), which is bound to its ligand to only 10% (Reul and De Kloet 1985; Reul et al. 1987). High tissue specificity for binding capacity of both

receptors can be considered as the MR/GR ratio differs markedly between central regions. Further the availability of GCs in the brain is limited by CBG, multidrug resistance P glucoprotein (MDR PGP) and the co-localization of 11-β steroid dehydrogenase type 2 (11βHSD2) in MR tissues selectively sensitive to aldosterone (De Kloet and Derijk 2004). The latter irreversibly converts cortisol and CORT to their MR/GR inactive 11 keto forms (De Kloet et al. 1998) and has been shown to promote aldosterone sensitivity in neurons of the nucleus tractus solitarius (NTS) (Geerling and Loewy 2006). MR binds to aldosterone with equal affinity as for CORT or cortisol (Dallman et al. 1989) and is involved in the regulation of salt and water homeostasis. In the periphery it is expressed in a variety of tissues like colon, lung, sweat and salivary glands, where 11βHSD2 is abundantly expressed but it has also been found without 11βHSD2 co-localization for example in mononuclear leucocytes, the heart, endothelial and epithelial cells and adipose tissues (Viengchareun et al. 2007). Like GR, the MR acts as transcription factor when activated by its ligand and in turn can be changed in expression by altered HPA axis activity (Karssen et al. 2007; Patel et al. 2008). In the brain, the MR is expressed in some aldosterone sensitive tissues (without co-expression of GR in the SCN) (Nader et al. 2010) and is co-expressed with GR in PVN as well as SO in humans (Wang et al. 2008), whereas in rodents results for co-localisation of both receptors in hypothalamic nuclei are inconsistent (Arriza et al. 1988; Ahima et al. 1991; Oitzl et al. 1995). Other prominent MR expressing structures are hippocampus (HC), amygdala (AG) and prefrontal cortex (PFC) (Reul and de Kloet 1986; Arriza et al. 1988; Ahima and Harlan 1990). Due to its high affinity to CORT and cortisol and its occupation by minimal daytime levels, one of the MR's main functions had been considered to be the 'tonic inhibition' of basal HPA axis activity (De Kloet and Reul 1987; Reul et al. 2000). In the line of this, elevation of baseline CORT levels in rats have been reported after intracerebroventricular (i.c.v.) (but not subcutaneous) or intrahippocampal administration of a MR specific antagonist (RU 28318) in several studies (Ratka et al. 1989; Oitzl et al. 1995; van Haarst et al. 1997). On the other hand, there is evidence for enhanced HPA axis reactivity to restraint stress by blocking the MR with RU 28318 or oligonucleotides (Ratka et al. 1989; Reul et al. 1997), which indicates that the MR plays a role in the acute stress reaction. The effect of habituation to restraint stress is erased by administration of RU 28318 but not spironolactone (Cole et al. 2000). Moreover, during the first restraint RU 28318 is unable to block rising levels of CORT but seems to be efficient in the response to a milder stressor – the CORT reaction to a novel environment (Cole et al. 2000; Pace and Spencer 2005). In humans, pharmacological studies, using either spironolactone or canrenoate as MR antagonist have repeatedly shown increment in cortisol secretion after administration (Born et al. 1991; Dodt et al. 1993; Deuschle et al. 1998; Young et al. 1998; Arvat et al. 2001; Young et al. 2003), even at circadian peak levels (Young et al. 2003) or after a combined

dexamethasone (DEX) / CRH test (Heuser et al. 2000). Other studies report no effect on cortisol of MR antagonists alone (Michelson et al. 1994; Mattsson et al. 2009).

2.2.3 Rapid effects of GC signaling

In addition to their genomic action, MR and GR have rapid effects on stress responsiveness which, in turn, can affect GC secretion within seconds. First evidence for rapid non-transcriptional mediated effects was already reported in the 1940s (see Dallman and Yates 1969) but has not been investigated as much as the transcriptional and transrepressive activity of steroid receptors which will be referred to later. Dallman and colleagues first reported fast feedback inhibition of stimulated HPA activity and subsequent *in vitro* studies further supported fast feedback inhibition on the release of CRH and ACTH (Widmaier and Dallman 1984; see Makara and Haller 2001 for a review). In parvocellular neurons of the PVN a rapid suppression of excitatory glutamatergic inputs by dexamethasone (DEX) and CORT could be antagonized by a cannabinoid receptor antagonist, elucidating downstream compensatory mechanisms (Di et al. 2003). In SON, DEX increases mini inhibitory post synaptic current (mIPSC) frequency within 3-5 minutes after administration *in vitro* and conversely decreases the frequency of mini excitatory post synaptic currents (mEPSC). In the same time, it has effects on the amplitude of electric stimulated currents, thereby enhancing electric (e) IPSC amplitude and diminishing eEPSC amplitude. This indicates a facilitation of presynaptic release of gamma amino butric acid (GABA) and suppression of glutamate release. In contrast to the GC induced suppression of glutamate release, facilitation of GABA secretion is not influenced by blockade of the cannabinoid receptor type I (CB1) (Di et al. 2005; Di et al. 2005; Di et al. 2009).

Effects for rapid signaling have been found for both GR agonists (Di et al. 2003; Atkinson et al. 2008) and aldosterone as MR agonizing substance (Atkinson et al. 2008). It had been proposed that there may be a modified version of GR capable to migrate into the membrane (Yudt and Cidlowski 2001) but evidence for either membrane bound GR as well as MR is still lacking (Huang et al. 2006; Viengchareun et al. 2007). Nonetheless, there is evidence for rapid MR mediated feedback inhibition of HPA activity by the MR agonizing aldosterone and moreover for disinhibition of CORT secretion by a MR antagonizing substance in female rats. Interestingly, only a single pulse of CORT secretion seems to be diminished in magnitude by the administration of aldosterone (Atkinson et al. 2008). Further, synaptic excitability of hippocampal cornu ammonis, subfield 1 (CA1) neurons seems to be dependent on MR rapid effects and are assumed to be mediated by pre- and postsynaptic membrane bound MRs. Effects of high GC levels on synaptic excitability can be observed within five to ten minutes after administration in vitro and protein synthesis inhibitors cannot block the MR related effects of high cortisol levels on the increase of excitatory glutaminergic transmission and the

decrease of post-synaptic hyperpolarization (Karst et al. 2005; Olijslagers et al. 2008). These findings underpin the assumption of non-genomic actions of cortisol on neuroendocrine, emotional and cognitive processes, as can be observed in inbred mouse strains differentially expressing MR and GR in suprahypothalamic structures (Brinks et al. 2009; Brinks et al. 2009).

2.3 Neurocircuitry of Stress

Short term exposure to circulating GCs can be beneficial or harmless but prolonged periods of high circulating cortisol levels alter HPA axis function and can have detrimental effects on different systems. While the whole organism has to adapt to diurnal and minute-to-minute variation in GC exposure, stress responsiveness may differ in a circadian rhythm as shown in a comprehensive meta-analysis for laboratory stressors in humans (Dickerson and Kemeny 2004). In the following, three suprahypothalamic structures will be highlighted that have systematically been investigated for their relation to HPA axis functioning and the effect of GCs on cognition. All of them co-express MR and GR, which are considered to mediate short and long-term effects of GCs on emotion, cognition and memory.

2.3.1 Hippocampus

The most prominent structure to exert negative HPA axis feedback via GR mediated mechanisms is the hippocampus (HC) (Sapolsky et al. 1986; Jacobson and Sapolsky 1991; Herman and Cullinan 1997). As a highly malleable structure, the HC undergoes a number of adaptive changes in response to acute and chronic stress. In humans, structural magnet resonance imaging (MRI) consistently show bilaterally reduced HC volume in the stress related disorders depression (Videbech and Ravnkilde 2004), probably mediated by childhood trauma (Vythilingam et al. 2002) and post traumatic stress disorder (PTSD) (Smith 2005). For PTSD, in turn, genetically determined smaller hippocampal volume is supposed to enhance the vulnerability for traumatic events, as has been shown in a study with trauma affected and unaffected monozygotic twins with or without PTSD (Gilbertson et al. 2002). Additionally, associations between low self-esteem as well as enhanced cortisol reaction to a psychosocial stressor (the *Montreal Imaging Stress Test* – MIST, a modified version of the *Trier Social Stress Test* – TSST see below) and reduced hippocampal volume have been shown with functional MRI (fMRI) (Prüssner et al. 2005). The TSST is a frequently used psychosocial laboratory stressor, consisting of mute preparation to and subsequent performance of a fictional job interview and a mental arithmetic task, both to be performed in front of a socially unresponsive panel (Kirschbaum 1993). Adapted to the MRI scanner situation, the MIST is based on an arithmetic task, operationalized in a way that participants can only reach 20 to 30% correct answers. Before the task starts, subjects are told that an

average college student reaches 80 to 90% correct answers and feedback about the percentage of correct answers is given constantly during the task (Prüssner et al. 2004). Increasing levels of cortisol were found to predict hippocampal atrophy as well as memory deficits in a long term study about aging (Lupien et al. 1998). Further, subjects that respond with increasing cortisol levels to the MIST show a relative HC inactivation during the task compared to non-responders, what indicates that HC driven negative HPA feedback hampered in MIST responders (Prüssner et al. 2008). This underpins early findings from animal lesion studies that repeatedly reported enhanced basal GC secretion and prolonged responses to several stressors including restraint (Sapolsky et al. 1986; Herman et al. 1995), contextual conditioning (Kant et al. 1984), acoustic stimulation (Nettles et al. 2000) and open field exposure (Herman et al. 1998). It can be assumed that HC driven negative feedback is specific for stimuli that are not physically harmful and seems to be mediated by a restricted set of neurons in the ventral subiculum and project to the bed nucleus of the stria terminalis (BNST) and hypothalamic neurons transmitting information to the PVN (Herman et al. 2003). The density of MR and GR in HC is considerably different between species. Whereas in the rodent brain GR is abundantly expressed (Reul and De Kloet 1985; Jacobson and Sapolsky 1991), it shows substantially lower expression in the primate brain. MR, on the other hand shows a notable density in the hippocampal structure in the rhesus monkey and human brain (Seckl et al. 1991; Sanchez et al. 2000). For both receptors expression changes after acute or chronic stress paradigms have been shown. Forced swimming, for example and the exposure to novelty can transiently increase the expression rate of MR in all hippocampal subfields in rats (Gesing et al. 2001) and GR expression is enhanced in CA1 in squirrel monkeys after the exposure to repeated social isolation in adulthood (Patel et al. 2008).

In rodents as in humans, learning and memory are affected by rising levels of cortisol in form of an inverted U-shaped curve, indicating that memory consolidation is impaired by too high or by insufficient levels of GCs. Physiological approaches have concentrated on investigation of long term potentiation (LTP) in rodents leading to the observation that the biphasic modulation of memory processes is mirrored by the impact of corticosteroids on LTP in the hippocampus (Lupien and Lepage 2001). Results of in vitro studies demonstrate that LTP is optimally induced by mildly enhanced GC levels or full MR occupation and some occupation of GRs. In the complete absence of adrenal steroids because of adrenalectomy, only poor LTP is detectable documenting that optimal LTP is MR dependent (de Kloet et al. 1999). This was further confirmed by studies with selective pharmacological blockade of the steroid receptors. Again, region specificity can be assumed because activation of MR promotes LTP in CA1 and in dentate gyrus of adrenalectomized (ADX) rats, while in CA3 MR has no effect on LTP. GR activation, on the other hand favors LTP in CA3 and suppresses it in CA1 and dentate gyrus (Pavlides et al. 1995; Pavlides et al. 1996; Pavlides and McEwen 1999).

Notably, exposure to a novel situation can inhibit LTP and even reverses earlier elicited LTP - an effect that seems to be associated to an acute stress reaction (Diamond et al. 1994; Xu et al. 1998). Other experiments show that in novelty detection of either spatial information or object exploration long term depression (LTD) is sensitive to the kind of stimulus in a region dependent manner (CA1: orientation cue → induction of LTD; DG: novel object → induction LTD) (Kemp and Manahan-Vaughan 2004; Kemp and Manahan-Vaughan 2008). There are many correlative findings that underpin the assumption that GC signaling by MR and GR can influence neuronal plasticity in HC. As reviewed by Bruce McEwen in 2007, adrenal steroids exert an influence on neurochemical systems that have been linked to atrophy of hippocampal neurons, dentritic retraction or changes in dendritic branching and neurogenesis on the other side (McEwen 2007). If chronic GC exposure really causes loss of hippocampal neurons, as it was formulated in the *Glucocorticoid Cascade Hypothesis* (Sapolsky et al. 1986), today can be doubted. Nonetheless it can be assumed that chronic stress leads to dentritic retraction of pyramidal neurons in hippocampal subfield CA3 and consequently can lead to increased vulnerability of neurons to additional neurotoxic events like metabolic challenges (Conrad 2008). For the impact of GCs on the development of hippocampal neurons there seem to be critical periods throughout life during which chronically enhanced levels of can have long lasting effects on atrophy of CA3 pyramidal neurons (Lupien et al. 2009). These consequences of chronic stress are considered to be mediated by enhanced extracellular levels of the fast excitatory neurotransmitter glutamate and downstream molecular pathways that are linked to chronic psychosocial stress (McEwen and Gianaros 2010).

2.3.2 Amygdala

Human fMRI studies revealed an association between high cortisol daytime peak levels and less activation of the amygdala following presentation of emotionally negative stimuli (Urry et al. 2006; Cunningham-Bussel et al. 2009) as well as deactivation of amygdala in subjects responding to the MIST compared to non-responders (Prüssner et al. 2008). This indicates that amygdala may play a role in acute stress regulation but also that there might be interindividual differences in the perception of potentially stressful stimuli.

In contrast to the HC, the amygdala (AG) rather stimulates HPA axis than to inhibit the stress response. The impact of AG on ACTH and GC secretion thereby is mediated by subnuclei in medial (MeA), the central (CeA) and the basolateral amygdala (BLA) that have distinct roles in HPA axis integration as they seem to react to different stimulus modalities. CeA is merely responsive to physically harmful stimuli like hemorrhage , cytokine infusion or lithium chloride infusions (Herman et al. 2003). This HPA stimulating effect is thought to be mediated by c-fos (an immediate-early gene) expression in the PVN which is increased after CeA

provocation by aforementioned stressors but not by psychological stressors like fear conditioning (Pezzone et al. 1992; Campeau et al. 1997). Sustaining the assumption of region specificity of the AG on HPA axis outcome, induction of c-fos by MeA is observed after exposure to psychological stimuli like restraint (Cullinan et al. 1995; Dayas et al. 2001), novelty (Emmert and Herman 1999) or fear conditioning (Pezzone et al. 1992) and is less pronounced after hypovolemia (Thrivikraman et al. 1997), cytokine stimulation (Sawchenko et al. 1990) or ether inhalation (Emmert and Herman 1999). As stress-regulatory structure, the basolateral amygdala has received considerable attention, consentingly activated by emotional stimuli. Its role in mediating HPA axis activity seems to be rather complex, when taking lesion study results into account that show no influence of BLA lesions on CRH, ACTH or GC secretion. On a behavioral level, on the other hand, BLA appears to be critical in the integration of memory consolidation of stressful stimuli (Roozendaal and McGaugh 1997; McGaugh and Roozendaal 2002) and seems to be important for the sensitization of HPA axis to the repetition of stress eliciting stimuli (Bhatnagar and Dallman 1998). As mentioned above, chronic (restraint) stress can trigger retraction of dendrites in CA3 but also causes dendritic growths in the BLA (Vyas et al. 2002) and shorter duration of the same stressor fails to elicit dendritic branch remodeling but still increases density of dendritic spines (Vyas et al. 2006).

BLA receives sensory input from thalamic and cortical thalamic pathways and mostly projects intra-amygdalar. BLA extensively innervates MeA and motivationally relevant sensory signals are projected to CeA, which can be considered as the primary output for behavioral and physiological adjustments. The CeA in turn, is the primary region mediating the induction of adaptive behavior or physiological adjustments by signaling to the PVN and lateral hypothalamus and to periaqueductal, medullary, and pre-autonomic nuclei via the stria terminalis. Further, the CeA is interconnected with prefrontal structures including the anterior cingulate cortex (ACC), ventromedial and orbitofrontal PFC (Herman et al. 2003; McEwen and Gianaros 2010). Interestingly, MR and GR distribution in the AG is region specific, as it is in the HC subregions. Most studies investigating the expression of brain GC receptors merely concentrate on HC, PFC, hypothalamic nuclei or pituitary but Patel and co-workers found that GR mRNA levels are higher in lateral than in medial AG whereas MR shows an inverse pattern of expression in the squirrel monkey brain (Patel et al. 2000). About the expression of MR in the human AG, still there is not much known. At least there is evidence for GR expression in human AG and furthermore that there are changes in its expression rate in major depressive disorder (Alt et al. 2010).

2.3.3 Prefrontal Cortex

The prefrontal Cortex (PFC) is the evolutionary and organizational highest brain structure and is known to be strongly involved in cognition, emotion and memory through neurons that maintain information in the absence of environmental stimuli. It is the key structure for the promotion of task relevant behavior and the inhibition of inappropriate actions. Under stressful conditions, functions of the PFC are impaired for example in working memory (Qin et al. 2009) and this can happen at GC levels that still enhance HC or AG driven memory (Murphy et al. 1996; Arnsten and Goldman-Rakic 1998). Functional neuroimaging studies have shown relative deactivation of PFC subfields in stressed subjects compared to non-stressed including the oribitofrontal PFC and the cingulate cortex (Wang et al. 2005; Prüssner et al. 2008) but findings are inconsistent regarding the pattern of activation/deactivation and correlation with cortisol levels. Taken together, fMRI data indicate that subregions of the PFC are involved in stress related cognitions and may regulate HPA axis outcome in a stressor and presumably sex specific (Wang et al. 2007) manner. An important mediator of this effect seems to be the controllability of the stressor. Amat and co-workers showed that the same stressor can produce a response of the nuclei raphé, when incontrollable which in turn is inhibited by the ventromedial PFC (vmPFC) if the stressor is controllable (Amat et al. 2005). Further, the vmPFC can modulate the activity of the AG in the consolidation of fear extinction (Quirk and Mueller 2008) In humans, fMRI studies show enhanced activity of vmPFC, and/or decreased activity in the AG during the inhibition of negative affect (Beauregard et al. 2001; Levesque et al. 2003; Ochsner et al. 2004; Ochsner et al. 2009) and further this pattern of activation is linked to 'normal' daytime cortisol levels, characterized by high morning and low evening cortisol (Urry et al. 2006).

Presence of MR and GR in PFC is well documented and has been studied in human post-mortem tissue or specimen taken after neurosurgery of patients with focal epilepsy. In PFC like in HC, sex, age and region specific distribution of both receptors have been reported (Watzka et al. 2000). In dorsolateral PFC MR expression has been shown to be lower in patients with bipolar disorder in all cortical laminae, whereas in schizophrenia a downregulation of MR seems to be restricted to layer I, III, IV and VI (Xing et al. 2004). Strikingly, expression changes of MR (among other genes) have been observed in vmPFC of squirrel monkeys which had been exposed to an adult social stress paradigm (Karssen et al. 2007; Patel et al. 2008). These results of differential MR expression in psychiatric disorders as well as in reaction to adult stress in non-human primates make abundantly clear that the MR is likely to play a role in stress related cognition.

2.4 Stress and Cognition

In the previous section the influence of suprahypothalamic brain regions on Cortisol secretion has been emphasized thereby shortly addressing the function of those regions in behavior and cognition. In the following, the reciprocity of the connection between stress and cognition will be highlighted by describing behaviors and cognitive processes that are affected by GCs. Learning and memory are important prerequisites for the interpretation of potentially stressful situations and the coping with stress. As mentioned earlier, a physiological stress reaction is elicited by situations characterized by novelty and unpredictability – in other words: by situations, in which an individual has not yet learned that he or she is capable to cope with. Once the unfamiliar characteristics have been learned and can be memorized, similar situations do not provoke a stress response anymore. This effect of habituation can be illustrated by the TSST, which in a large percentage of subjects triggers a two- to threefold rise in cortisol only the first time. In about half of the participants, HPA axis reactivity diminishes when the TSST is repeated (Kirschbaum et al. 1995; Schommer et al. 2003). In turn, cortisol has the potential to alter memory processes as has been shown in numerous investigations in animals as well as in humans. Referring to the abundant literature about rodent learning and memory it now can be concluded that the either beneficial or impairing effects of stress depend on the source of the stressor, the duration of stress (acute vs. chronic), its intensity, the stressor timing with regard to the memory phase (acquisition, consolidation, retrieval) and the type of learning as described in a comprehensively structured review (Sandi and Pinelo-Nava 2007). If GCs thereby have beneficial effects on memory formation is assumed to be dependent of the coupled incidence of the stressor, the event to be remembered, and the coincidence of stress hormones and neurotransmitters with the neuronal circuits activated by the material that should be learned (Joels et al. 2006). In humans, a prominent finding is the impairment of explicit memory and working memory after administration of synthetic GCs or the application of the TSST (Newcomer et al. 1994; Kirschbaum et al. 1996; Lupien et al. 1999; Newcomer et al. 1999; de Quervain et al. 2000; Luethi et al. 2008). The association of GCs and memory has been found to be mediated by the emotional quality of the material to be remembered, indicating an advantage of stress on memory of (negatively) arousing material over neutral stimuli (Buchanan and Lovallo 2001; Cahill et al. 2003; Tops et al. 2003; Putman et al. 2004; Payne et al. 2007; Luethi et al. 2008). Effects of GCs on memory for emotionally salient stimuli may further be moderated by additional factors. Memory for negative stimuli, for example, has been shown to be ameliorated only in men that showed enhanced negative affect related to a psychosocial stressor (Abercrombie et al. 2006). Traditionally, the effects of GCs on memory had been associated to the HC and therein to GR mediated functions because they appear at enhanced levels of GC secretion or administration of GR occupying doses of GCs (reviewed

in Lupien and Lepage 2001). Indeed, GR polymorphisms modulate the impact of acute cortisol administration on working memory (Kumsta et al. 2010). However, GR levels in primate HC seem to be underrepresented compared to rodents but the receptor is highly expressed in PFC (Sanchez et al. 2000) and memory functions (particularly working memory) can be attributed to PFC activity (Roozendaal et al. 2004).

High levels of circulating cortisol have been associated with other cognitive processes than memory and the literature about the effects of acute or chronic stress on cognitive processes in humans (particularly in healthy populations) is growing. Prolonged administration of cortisol has been shown to impair spatial working memory and pattern recognition, the latter being assumed as a HC unrelated form of memory (Young et al. 1999). Single dose administration of cortisol as well as acute psychosocial stress have been shown to be associated with elevated acoustic reflex thresholds (Fehm-Wolfsdorf et al. 1993), which is considered as a rapid mechanism of acoustic attention. Furthermore, cortisol secretion after acute psychosocial stress is linked to greater attentional inhibition (Skosnik et al. 2000), enhanced vigilance in an emotional stroop task (van Honk et al. 2000), emotional information processing (Ellenbogen et al. 2002; Ellenbogen et al. 2006), and approach-avoidance behavior (Roelofs et al. 2005). Anticipatory primary appraisal can be considered as predictor of the TSST stress response (Gaab et al. 2005) and social cognition is associated with the magnitude of the TSST response, characterized by inversed correlational patterns in men compared to women (Smeets et al. 2009). Effects of cortisol on cognition can be considered as context dependent and they are linked to subclinical or clinical symptomatology like depression or anxiety (Ellenbogen and Schwartzman 2009; Roelofs et al. 2009). Due to the complex neurocircuitry of stress and the difficulty to set up thresholds for MR and GR occupation in the related brain areas, it will be a substantial challenge to shed light on the role of distinct brain regions or circuits in stress related cognition.

2.4.1 The Mineralocorticoid Receptor in Stress Related Cognition

In contrast to the GR whose function in memory acquisition and consolidation has exhaustively been investigated, the role of MR in cognitive functions still remains 'mysterious' (Reul et al. 2000) – at least in human research. It is clearly easier to describe the function of a system that acts in extraordinary situations then to reveal the characteristics of a 'steady-state' system that seemingly only exhibits minimal variation.

Via selective pharmacological blockade at different stages of spatial navigation in the Morris Water Maze, it has early been shown that MR blockade leaves acquisition and consolidation of spatial memory intact but changes the search-escape strategy. These behavioral changes were characterized by less anxious swim patterns during free swim trials, when the escape platform is removed (Oitzl and de Kloet 1992). Thereafter, the same research group showed

that a naturally hippocampal MR overexpressing rat strain, the Lewis rat, is ACTH and CORT hyporesponsive to a novel environment as well as an interleukin-6 (IL-6) challenge and attenuated ACTH levels after tail nick (but a comparable CORT reaction). Still, Lewis rats had lower levels of ACTH and CORT at the diurnal peak, which could be elevated by i.c.v. injection of a MR antagonist (Oitzl et al. 1995). Furthermore, in studies in two inbred mouse strains (BALB/c and C57BL/6J) behavioral differences have been described in respect to different MR and GR expression in HC, AG and PFC. Increased stress vulnerability of BALB/c mice can be ascribed to lower hippocampal expression of both receptors but increased levels of GR mRNA in the PFC and GR protein in the amygdala and is associated with high emotional expression and spatially orientated cognitive performance. In C57BL/6J mice, which show high levels of MR and GR, cognitive performance is rather stimulus driven and they show lower vulnerability to stress (Brinks et al. 2007).

Transgenic rodent models

To date, findings on MR affected behavior stems also from genetically modified rodent models. Complete, comprising central and peripheral knock-out of MR leads to fatal pseudohypoaldosteronism that can be adjusted by exogenous NaCl administration, but the model is hard to interpret because of the peripheral effects of this method (reviewed in Kolber et al. 2008).

First promising mouse models for the investigation of central MR function comprised the specific depletion of MR in limbic structures by a combined loxP/CaMKIIa regulated Cre recombinase approach that results in mice completely lacking MR in limbic structures, as assessed by immunohistochemistry (Karst et al. 2005). Another method is to overexpress MR under the control of calcium/calmodulin dependent protein kinase type II (CaMKII) promoter. This technique causes an excess in MR expression in HC and forebrain, leaving levels in the PVN comparable to wild-type controls (Lai et al. 2007; Rozeboom et al. 2007). Further, a herpes simplex based vector, expressing MR, can be infused in different tissues, changing MR levels locally as has been shown for the hippocampal formation and BLA (Ferguson and Sapolsky 2008; Mitra et al. 2009). In a first study, using transgenic mice that have a specific MR knock-out in limbic structures, results indicate that the MR plays a crucial role in the rapid non-genomic effects of GCs.

In CA1 cells, stemming from forebrain MR lacking mice, application of CORT did not elicit an increased frequency of miniature excitatory postsynaptic currents in contrast to cells taken from forebrain GR knock-out mice within five to ten minutes after agonist application (Karst et al. 2005). It remains to be said that another study, using the same MR forebrain knock-out technique reports no effect of MR on CA1 neuron excitability but reveals changes in HC mossy fiber projections as well as an enhanced expression of GR in HC cornu ammonis

(CA). This finding has been interpreted as compensatory mechanism to the loss of MR (Berger et al. 2006). Nonetheless, forebrain MR overexpressing mice show reduced neuronal loss after transient global cerebral ischaemia (Lai et al. 2007), indicating a role of MR in neuron survival.

Behavioral effects of altered MR expression

Whereas the first study working with forebrain specific knock-out of MR concentrates on functions of the MR in rapid GC signaling, further studies rather focus on the behavioral changes that accompany expression changes of MR in the forebrain. Already in 2006 a study was published using the same transgenic mouse model to describe more passive behavior of forebrain MR lacking mice in the *Morris Water Maze* (MWM) and the eight arm radial maze. The transgenic mice (MR^{CaMKCre}) learn and reacquire platform positions in the MWM but show an overall poorer performance then wild-type controls. MR^{CaMKCre} mice generally show more reentries in arms already visited in a baited eight-arm radial maze (Berger et al. 2006). This finding may indicate that MR is implicated in reward related actions with a higher tendency of MR^{CaMKCre} to repeat formerly rewarded behavior. Interestingly, MR^{CaMKCre} show increased exploration of novel objects added to a familiar environment (Berger et al. 2006), supporting the assumption that loss of functional MR in central structures impairs behavioral flexibility rather then affecting processes of learning and memory.

In anxiety related measures as the open field test, an elevated O maze and the dark-light box, MR^{CaMKCre} mice don't differ from control mice in this study. Further, MR^{CaMKCre} and control mice were indistinguishable in two forms of associative learning as assessed by a shock-avoiding paradigm in a two chamber box and taste aversion learning (Berger et al. 2006). A subsequent investigation focusing on emotional arousal and fear memory shows that application of prior restraint stress leads to more passive behavior in a hole board (used for comparable measures as an open field) in MR^{CaMKCre} mice compared to stressed controls. During cue and context related fear conditioning, MR^{CaMKCre} mice exhibit significantly more freezing than control mice for memory testing (cue and context without shock exposure) and during assessment of the level of extinction. These differences are mainly due to more freezing of MR^{CaMKCre} during contextual episodes showing that MR^{CaMKCre} don't differentiate between conditioned cues and context in contrast to control mice, who display less freezing to context episodes, after extinction had happened (Brinks et al. 2009).

In two different laboratories but at the same time, limbic MR overexpressing mouse strains (MRov) were elevated to further characterize MR functions. Both strains show a reduction of anxiety related behavior as shown by a reduced latency of visits in the dark compartment in a dark-light box and a higher percentage of time spent in the center of the open field (Lai et al. 2007; Rozeboom et al. 2007) and more time spent in the open arms of the elevated plus

maze (Rozeboom et al. 2007). Forebrain MR overexpression further changes the behavior towards a novel object. Contrarily to wild-type mice, MRov spent longer time per visit at the novel object but visit old and new object equally frequent. In the MWM MRov spend more time in the former learned quadrant when the platform is removed, indicating a stronger learning of the former position (Lai et al. 2007). The results show a high level of consistency even if the vector infusion led to different patterns of MR overexpression. Most importantly, one group found higher levels of MR mRNA in the BLA of treated mice (Lai et al. 2007) while the other group finds no difference between control mice and MRov (or MRTg in their terminology) in AG. In the group without MR overexpression in AG, enhanced levels of the serotonin transporter 1a (5HTT-1a) are found in CA1 as well as increased levels of GR mRNA, indicating compensatory changes in HC function (Rozeboom et al. 2007).

Another strategy – the local increase of MR via injection of a MR expressing vector - has been used by the research group of Robert Sapolsky. Injected in the HC in adrenalectomized rats, it increases the amount of MR locally and was shown to have beneficial effects on novel object recognition 24 hours after object learning. Administration of high doses of CORT before memory retrieval further facilitated novel object recognition in animals treated with the MR containing vector (Ferguson and Sapolsky 2007). In this study, there was no effect of MR overexpression on short term memory but a further study of this group showed that short term memory effects are mediated by GR by injecting a negative transdominant vector GR alone or in combination with MR (Ferguson and Sapolsky 2008). Overexpression of MR in the BLA using the same method, resulted in a reduction of CORT levels after restraint stress transiently and decreased anxiety as assessed by the elevated plus maze and the open field test (Mitra et al. 2009).

Results of transgenic mouse models are summarized in Table 2.1 and for studies investigating post-natal MR depletion in limbic structures in rats in Table 2.2. The Tables give an overview about affected brain regions, alternations of HPA axis function as well as affected behaviors and changes in neuronal plasticity or consecutive molecular changes.

MR mouse line	tissues affected by knock-out	HPA regulation	Behavior	neuronal outcome
conventional MRKO	All MR expressing tissues → develop PHA, rescued by exogenous NaCl	Elevated CRH in PVN Increased POMC & ACTH in pituitary Increased levels of CORT		Decreased granule cell density in HC

MR^{CaMKCre} (Karst et al. 2005)	Loss of MR in all limbic neurons	No HPA changes in CORT levels at diurnal peak & trough or after restraint		Induction of mEPSC in CA1 cells by aldosterone → fast onset, rapidly reversible
MR^{CaMKCre} (Berger et al. 2006)	Loss of MR in all limbic neurons	No HPA changes in CORT levels at diurnal peak & trough or after restraint	Impaired learning in MWM Perseverations in radial maze Passive behavior Increased reactivity to novel object	Upregulation of GR in HC Changes in HC mossy fibers
MR^{CaMKCre} (Brinks et al. 2009)	Loss of MR in all limbic neurons	No HPA changes in CORT levels at diurnal peak & trough or after restraint Increased CORT levels in fear conditioning (after extinction trials)	Passive behavior in unbaited hole board (stressed) Reduced signs of fear extinction → more freezing to context than controls	
MrTg (Lai et al. 2007)	Overexpression of MR in HC, cortex, striatum, lateral septum and BLA	No HPA changes in CORT levels at diurnal peak & trough or after restraint	Decreased anxiety in open field and light-dark box Altered exploration of novel objects Increased spatial learning in MWM	Attenuation of neuronal damage after ischaemia
MRov (Rozeboom et al. 2007)	Overexpression of MR in HC, cortex	No HPA changes in CORT levels at diurnal peak & trough Female mice: diminished CORT levels after restraint	Decreased anxiety in open field and elevated plus maze	Decreased GR levels in CA1 Upregulation of 5HT-1a in CA1

Table 2.1: Overview inbred MR transgenic mouse lines, MR expression changes are persistent.

MR rat line	tissues affected by knock-out	HPA regulation	Behavior	neuronal outcome
MRMR HC overexpression (Ferguson and Sapolsky 2007)	HC (dental gyrus)	Normalized via ADX & low vs. high CORT pellet	Enhanced non-spatial novel object recognition (long term) Attenuation of impairing effects of high CORT on short term object placement memory	
MRMR BLA overexpression (Mitra et al. 2009)	basolateral amygdala	Reduced CORT after immobilization	Reduced anxiety in EPM (stressed and non-stressed) and in open field (stressed)	

Table 2.2: Overview MR rat lines, note that MR expression changes listed here are transient.

2.5 The Mineralocorticoid Receptor

2.5.1 The Mineralocorticoid Receptor Gene (*NR3C2*)

The MR is the member 2 of the nuclear receptor subfamily 3 (steroid receptors), group C (NR3C2) and shows high overall similarities with the GR (NR3C1) even if they are coded on different chromosomes. The GR gene is located on Chromosome 5q31.3 and MR is coded in Chromosome 4q31.1 (Morrison et al. 1990). Both receptors encompass nine exons that code for the same protein domains but on a transcriptional level, there is a higher variety of GR transcripts known today. Especially the 5' untranslated region in NR3C1 shows high variability, containing nine alternative exons 1 of which two can further be alternatively spliced, thus resulting in eleven different transcripts. Each of the first exons has an own promoter region accounting for tissue specific regulation of the different transcripts (Turner and Muller 2005; Turner et al. 2006; Presul et al. 2007). The whole MR gene spans approximately 450 kilo bases (kb) (Zennaro et al. 1995) and until now there are only two alternative exons 1 (α, β) and associated promoters found in the human MR (hMR). They lead to two different mRNA isoforms (hMR α, hMR β), both expressed in various human aldosterone target tissues while there exact function, presumably affecting transcript stability or translational efficiency remains to be determined. Both promoters have been shown *in vitro* to be activated by GCs and P2 (the distal promoter) additionally can be stimulated by aldosterone, in contrast to the proximal, normally stronger P1. Early findings in rats indicate at least one more alternative exon 1γ (Patel et al. 1989). The transcription initiation site is located two base pairs (bp) downstream of the beginning of exon 2 the coding region for most of the N-terminal domain (NTD) of the MR protein. Exons 3 and 4 build each one of the

zink fingers of the DNA binding domain (DBD) which enables the MR to activate or repress the transcription of other genes. The resting exons 5 to 9 code for the hinge region and the ligand binding domain (LBD) (Pascual-Le Tallec and Lombes 2005; Viengchareun et al. 2007). Highly conserved between species, there are two strong translation initiation sites that correspond to methionine 1 and methionine 15 in the MR protein amino acid sequence. It has been shown that they account for two different isoforms of the protein MR-A and MR-B that seem to have important phylogenetically conserved functions. Six additional weaker Kozak consensus sequences may lead to further shortened isoforms (Pascual-Le Tallec et al. 2004) but their expression pattern or function remains to be investigated. Apart from the alternative exons 1 and the two translation initiation sites, there are further splice variants which will be described below.
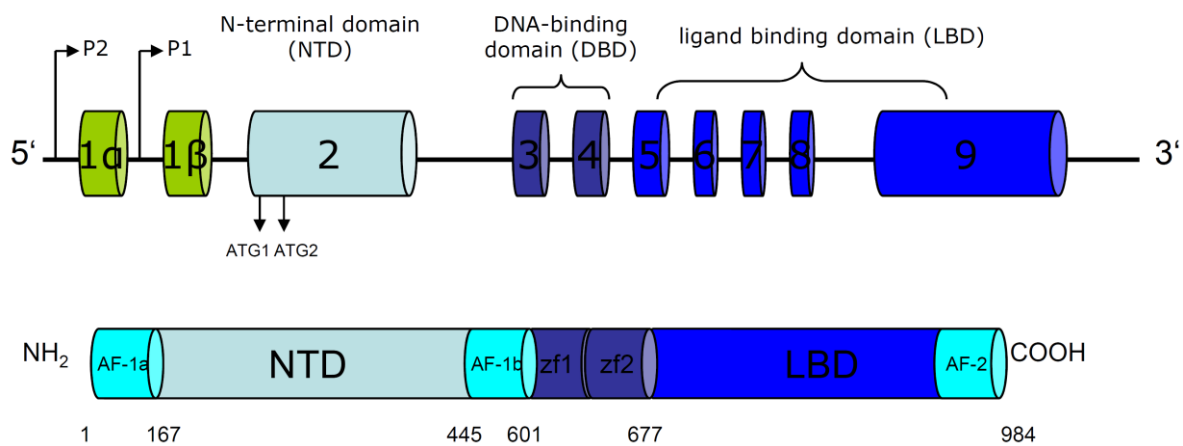
Figure 2.1: Structure of NR3C2 and the full length MR-A protein.

2.5.2 The MR Protein

In the case of transcription of either exon 1α or 1β, the same 107 kilo Dalton (kDa) translational product will be generated. This whole MR protein consists of 984 amino acids that are divisible in a 602 amino acids long NTD, the 66 amino acids long DBD that shows high similarity with GR (94%) and the progesterone and androgen receptor (more then 90%), a hinge region (~61 amino acids) and finally the complex and multifunctional LBD that spans 251 amino acids and is remarkably conserved between steroid receptors (55% homology) and species (80-97% orthology) (Pascual-Le Tallec and Lombes 2005; Viengchareun et al. 2007). Due to alternative translation start sites, it comes to the full length isoform MR-A and the 14 amino acids smaller isoform MR-B (105 kDa, 970 amino acids).

The length of the NTD differs a lot between steroid receptors but is relatively homologous between species (~50%) for a given steroid receptor what indicates crucial functional importance. The MR NTD is the longest of all steroid receptors and comprehends functional

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domains that account for ligand independent transactivational or transrepressive functions. These are exerted by two activation function I domains (AF-1a and AF-1b) as well as a central inhibitory domain that attenuates the transactivation strength of the NTD fused to either AF-1a or AF-1b (Tallec et al. 2003). These different domains of the NTD recruit a variety of co-activators or –repressors modulating the transcriptional activity of MR in a manner that is highly selective compared to other steroid receptors and seem to account for mineralocorticoid selectivity (Pascual-Le Tallec and Lombes 2005; Viengchareun et al. 2007).

The DBD of the MR consists of two zinc fingers of which the first one contains the 'P-box' that is important for a tight binding to the minor groove of the DNA double helix and the second facilitating dimerization via the so called 'D-box'. Heterodimerization of MR preferentially includes the GR or androgen receptor (Liu et al. 1995) and can be considered as mechanism for specific transcriptional regulation of physiological responses. In the DNA sequence coding for the DBD a 12-bp insertion has been identified that results of the use of a cryptic splice site at the splice junction on exon 3/intron C leads to the in frame insertion of four additional amino acid between the two zinc fingers of the DBD (Bloem et al. 1995). Expressed with different abundance within diverse brain regions (Bahr et al. 2004), this variant exhibits the same transcriptional activity with aldosterone as ligand as the 'wild-type' MR (Wickert et al. 2000). Two further isoforms that alter the function of the protein drastically stem from skipping of exon 5 alone or exons 5 and 6 what leads to mRNA isoforms Δ5 respectively Δ5,6 hMR. After translation, the Δ5,6 hMR (75 kDa) codes for a receptor that remains able to bind to DNA and acts in a ligand independent manner due to the depletion of the hinge region and LBD by a premature stop codon. It has been shown to modulate GR and MR transcriptional capacities (Zennaro et al. 2001).

Finally, the LBD transduces endocrine signaling into specific transcriptional responses, allowing selective hormone binding. It consists of 11 α-helices and four small anti-parallel β strands, resulting in a three layer helical sandwich. The LBD possesses an activator function 2 domain (AF-2), formed and activated in a ligand dependent manner by helices H3, H4, H5 and H12 after agonist binding into the hydrophobic pocket of the LBD (Pascual-Le Tallec and Lombes 2005; Viengchareun et al. 2007), thereby enabling the binding of coactivators as well as subsequent MR activation. Aldosterone as a ligand is bound in a fully enclosed pocket to residues of helices 3, 4, 5, 6, 7 and 11 and the β turn. (Bledsoe et al. 2005) Other functions encompass the nuclear localization, interaction with chaperones and dimerization (Yang and Young 2009).

2.5.3 Transcriptional Activity of the MR

In the absence of a ligand, the MR predominantly resides in the cytosol, building up a hetero-oligomer in interaction with various proteins. Prominent MR bound proteins are the heat shock proteins hsp90 and hsp70, the p23 and p48 proteins, the FKBP59 immunophilins or CYP40 cyclophilin as well as with actin (Binart et al. 1995; Jalaguier et al. 1996; Bruner et al. 1997; Pratt and Toft 1997). Binding of the ligand induces a conformational change resulting in dissociation of the MR chaperones, homo- or heterodimerization and the translocation to the nucleus where it binds to GC responsive elements (GREs) in the (distal) promoter region of other genes. There it directs the transcription of target genes, interacting with numerous molecular partners, most prominent transcriptional coregulators, either acting as coactivators or corepressors of the MR.

Translocation to the nuclear compartment is guided by three known nuclear location signals (NLS). The first one (NLS0) is located between amino acids 590 and 602 in the C-terminal region of the NTD, NLS2 lays in the LBD devoid of basic amino acids what is a known feature of steroid receptor NLSs. The third NLS (NLS1) is positioned in the C-terminal domain of the DBD near to a nuclear export signal residing between the two zinc finger motives. NLS0 seems plays a crucial role in nuclear import, which is dependent upon phosphorylation of Ser601. Ligand specifity is thought to be mediated by NLS2 based on the observation that MR agonists induce rapid receptor translocation whilst antagonists are less effective. NLS1 interacts with the other two thereby facilitating translocation of the MR into the nucleus (Lombes et al. 1994; Black et al. 2001; Walther et al. 2005).

Coactivators frequently bind to the AF-2 region of the LBD where they perform many of the enzymatic reactions that are needed for gene expression including chromatin remodeling, histone modification, initiation of transcription, elongation of RNA chains, RNA splicing, and termination of transcriptional responses, often recruiting further molecules in an ordered cyclical manner. The first indentified coactivator is the steroid receptor coactivator 1 (SRC-1) (Onate et al. 1995) and since then ten further coactivators (SCR-2, p300/CBP, PGC-1α, RHA, ELL, FLASH, FAF-1, Ubc9, TIF-1α, RIP-140) and five corepressors (SMRT, NCoR, DAXX, PIAS1, NF-YC) have been found (Yang and Young 2009). Results of studies assessing the binding of MR coregulators indicate that they are not specific for different MR ligands as tested for aldosterone, cortisol and CORT but that may be a side effect of the chosen assay technique (Lee et al. 1999; Li et al. 2005).

Corepressors induce histone deacetylation and other inhibiting mechanisms of gene transcription. They have been shown to bind to the LBD of the ligand unbound MR and are replaced by coactivators upon ligand binding (Viengchareun, 2007, YangYoung 2009).

Most of what is known about the transcriptional activity of the MR has been investigated relating to aldosterone as MR ligand because MR shows high selectivity for aldosterone

among the steroid receptors and so plays a crucial role in all aldosterone mediated processes. In order to identify target genes of GC induced MR, Datson et al. used serial analysis of gene expression (SAGE) in the HC of adrenalectomized (ADX) rats previously treated with different doses of CORT to disentangle genes specifically regulated by MR and GR. They identified 131 genes differentially regulated between ADX rats and ADX rats treated with low levels of CORT, accounting for an effect of MR on expression changes of which 76 were up-regulated and 55 down-regulated. Genes that showed altered expression are implicated in cellular metabolism and energy production, signal transduction, protein synthesis, trafficking and turnover leaving several genes with unknown functions. Between low and high CORT treatment, as an indicator for GR regulated genes resulted in identification of 105 tags, 44 up-regulated and 61 down-regulated. They fit in the same functional classes as MR controlled genes but individual genes belonging to a certain functional class were mostly different. Only 33 genes between the MR and the GR activating conditions were found to be regulated by both receptors (Datson et al. 2001).

2.5.4 MR polymorphisms

To date, 546 SNPs are listed for *NR3C2* in the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez). The most prominent finding for SNPs in *NR3C2* is that they mediate pseudohypoaldosteronism type 1 (PHA1), first described in 1858 (Cheek and Perry 1958). This disease is characterized by renal resistance to aldosterone actions and patients suffer from salt wasting, hyperkalaemia and metabolic acidosis despite increased serum aldosterone and renin levels. Two forms of PHA exist, one being autosomal dominant (adPHA1) and the other autosomal recessive (arPHA1) adPHA1 showing a milder course then the autosomal recessive form. Mice with a homozygous knock-out of *Nr3c2* show severe symptoms of PHA1 confirming the role of MR in this disease. About 50 distinct polymorphisms/mutations have been described for the MR that contribute to this syndrome and that include missense, nonsense, frameshift, and splice site mutations, as well as deletions spreading throughout the whole gene. In humans, only heterozygous occurrence has been found for various polymorphisms, indicating that homozygous expression of those the MR protein affecting polymorphisms may be lethal *in utero*. The polymorphisms accounting for PHA are characterized by a loss of MR function in terms of integration of translation stop signals or leading to a loss of functional domains (Viengchareun et al. 2007).

There are two polymorphisms in human stress research that have been investigated for their role on HPA axis outcome or behavioral variables. First, the single nucleotide polymorphism (SNP) with the rs number rs5522 had been investigated 2006 by our research group. The SNP is called MRI180V because the change from A→G on this locus leads to an isoleucine

to valine in the amino acid on position 180 of the protein. The SNP attenuates the transcriptional activity of the MR using cortisol as a ligand *in vitro* whereas by the use of aldosterone this effect was not observed (DeRijk et al. 2006). In a sample of young healthy male twins, carriers of the minor G allele (frequency ~12%) exhibited significantly higher salivary and plasma cortisol responses to the TSST as well as an increased heart rate. In contrast, no differences emerged in plasma ACTH. The effects lasted for two further repetitions of the TSST showing that both groups nevertheless habituated at an equal rate but overall higher responses of carriers of the minor MRI180V allele (DeRijk et al. 2006). The same SNP has been found to be linked to the emergence of depressive symptoms in the Leiden 85-plus Study in an elderly population. In this group, the SNP was not related to impairments in attention or processing speed and no association with overall cognitive measures has been observed (Kuningas et al. 2007). In this cohort an additional MR SNP had been assessed, not linked to depression or cognitive measures. The rs2070951 will here be referred to as MR-2G/C as it is located two basepairs downstream the translation initiation site and consists of a G to C transition with both alleles occurring frequently (G: 53%; C:47%). The G allele was found to diminish the transactivation capacity of the MR *in vitro* with cortisol as well as DEX used as ligand. Both SNPs, the MRI180V and MR-2G/C, have been shown to influence the suppression of the cortisol awakening response (CAR) after a low dose DEX administration the evening before in a sex dependent manner. The sample investigated was composed of 218 students, recruited from the Trier University with no prior history of disease or actual medication and they were non-smokers. All women used oral contraceptives, what may contribute to the observation that the effect of both SNPs on CAR suppression was bigger in men (van Leeuwen et al. 2010). Only very recently, the MRI180V was tested for its association with reward learning under stressful and non-stressed conditions, showing that reward learning is enhanced in non-stressed carriers of the minor G allele and that the stressful condition leads to an intensified impairment of reward-related behavior modulation in this group (Bogdan et al. 2010). In a previous study, the detriment of the ability to change behavior in response to reinforcement has been shown for depressed subjects as well as for patients with bipolar disorder (Pizzagalli et al. 2008; Pizzagalli et al. 2008). Together with the findings of the Kuningas study (2007), and considering that MR expression is altered in dorsolateral prefrontal cortex of patients suffering from bipolar disorder these results point towards a specific role of MR polymorphisms in the development of psychiatric disorders which is associated with specific cognitive impairment under the control of stress eliciting situations.

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CHAPTER 3

Associations between Mineralocorticoid Receptor Polymorphisms and accuracy in attention networks and set shifting

Chapter 3: Associations between Mineralocorticoid Receptor Polymorphisms and accuracy in attention networks and set shifting

Background: Pharmacological blockade of the MR has been shown to affect performance in selective attention, visuospatial memory and a set shifting task in humans. Recently, one SNP in *NR3C2* has been shown to modulate reward related adaption of behavior under stress. So, polymorphisms of the mineralocorticoid receptor (MR) genes might affect the same cognitive domains but effects are probably less pronounced.

Methods: We investigated 72 healthy subjects to assess associations between MR polymorphisms and attention (*Attention Network Task* – ANT) as well as a set shifting paradigm (*Wisconsin Card Sorting Test* – WCST, modified)

Results: The group with the most common genotype (MR-2G/C heterozygous, MRI180V A homozygous) has the lowest error rates in ANT conflict and alertness, followed by the MR-2G/C G homozygous subjects. G carriers of I180V and -2G/C C homozygous subjects are not significantly different one from the other and show the highest error rates. Accuracy in WCST is only affected on a trend level, showing differences in detection of intradimensional shifts in the WCST paradigm.

Conclusions: We found differences between MR genotype groups composed of young healthy subjects in behavioral measures of cognitive tests. These group differences indicate an association of MR polymorphisms and prefrontal executive functions. The role of MR polymorphisms in attention and set shifting are a striking finding, indicating an involvement of MR in a basic function of human cognition.

3.1 Introduction

Reviewing the literature on MR functioning in rodents reveals effects of the receptor on visuospatial memory assessed by behavioural measures in the Morris Water Maze, on anxiety measures, on the recognition of novel objects and on habituation to different stressors (Oitzl and de Kloet 1992; Cole et al. 2000; Pace and Spencer 2005; Berger et al. 2006; Lai et al. 2007; Rozeboom et al. 2007).

Translating these phenotypes and protocols to research in human subjects represents a substantial challenge. The literature on the relation between MR function and cognitive operations in humans is very sparse with only two studies assessing this association in young healthy populations. In the first study a pharmacological approach was chosen blocking the MR with spironolactone in the experimental group and a battery of different paper and pencil cognitive tests was carried out. The investigators found significant differences in selective attention/concentration as measured by the *d2' test* (Brickenkamp 1978). Further, the experimental group showed decreased performance in visuospatial nonverbal memory as well as a trend towards impaired cognitive flexibility (Otte et al. 2007). The second study comprises two sub-studies and is based on a candidate gene association approach. Subjects were genotyped for rs5522 or, in the further, MRI180V, a single nucleotide polymorphism (SNP) that leads to an isoleucine to valine change at position 180 of the MR protein. Participants were tested under basal, non-stressed conditions (study 1) and a stress condition, in which subjects attended to a threat-of-shock paradigm (study 2). The assessed behavior was the systematic preference or 'response bias' for rewarded stimuli in a probabilistic reward task. Carriers of the rare (G) allele of the polymorphisms showed enhanced reward learning and the generally impairing effect of stress on reward learning as measured in study 2 was larger for this group than for participants homozygous for the common (A) allele (Bogdan et al. 2010). Altogether, these two studies provide evidence for a relation between MR functioning and multiple cognitive domains. The most basic ability associated with MR functioning, assessed by the d2' test, might be attention to stimulus material of relative low complexity.

Attention has exhaustively been investigated since the early 1950s and today, the common sense is that there are at least three prominent functions of attention: orientation towards a sensory event, the detection of signals for conscious processing and the maintenance of a vigilant or alert state (Posner and Petersen 1990). An instrument to assess these different aspects of attention is the *Attention Network Task* (ANT) and it was constructed to measure three distinct neuronal networks that are activated independently to process different aspects of attention (Fan and Posner 2004; Raz 2004). The three components measured are "alertness", "orienting" and "conflict/executive control". As could be shown by fMRI, the three factors of attention have distinct neural correlates, building up three attention networks that

are activated largely independently of each other. The alertness network activates frontal and parietal areas predominantly on the right hemisphere as well as the thalamus. For the orienting network the left temporoparietal junction, frontal eye fields and areas in the superior parietal cortex have been described to be activated conjointly. For the execution network lateral ventral prefrontal areas and the anterior cingulate cortex have been shown to react with higher activation to conflicting material than to non-conflicting cues (Raz 2004; Fan et al. 2005). It has been proposed that acetylcholine acts as neuromodulator for the orienting network, norepinephrine for alertness, and dopamine for the executive control network. Heritability estimates assessed in a twin study comparing monozygotic and dizygotic twins (26 pairs each) did differ across the three attention networks. A substantial heritability could only been found for the executive control network (h^2_F= .89) while for alerting (h^2_F= .18) and overall reaction times (h^2_F= .16) estimates were moderate and no heritability was detected for the orienting network (h^2_F= -.59) (Fan et al. 2001). Due to the relatively small sample size, these results should be cautiously interpreted and can rather be described as exploratory. Polymorphisms in the genes for the dopamine receptor D4 (*DRD4*) and monoaminoxidase A (*MAOA*) were associated with the executive control index in genetic association studies and have further been shown to be linked with frontal brain activity for the congruent and incongruent condition of the "conflict" network (Fossella et al. 2002; Fan et al. 2003). In another study, an association with higher error rates in the conflict network has been reported for homozygous carries of the minor allele of a polymorphism in tryptophan hydroxylase 2 gene (*TPH2*). In the latter study, a SNP in the gene coding for catechol-O-methytransferase (*COMT*) was not associated with group differences in one of the attention networks (Reuter et al. 2007). For highly obese patients and patients suffering from post traumatic stress disorder (PTSD) impairments in the executive control network have been described (Beutel et al. 2006; Leskin and White 2007). Schizophrenic inpatients were found to be impaired only for the network of executive control compared to healthy controls in one study (Gooding et al. 2006) but showed additional impairment in the orienting network in another experiment with a larger sample size (Wang et al. 2005).

Other cognitive/behavioral domains that are related to MR functioning are visuospatial memory and/or navigation (e.g. Oitzl and de Kloet 1992; Otte et al. 2007), reward learning (Bogdan et al. 2010), and cognitive flexibility (Otte et al. 2007) or, as formulated elsewhere: "[…] MR activation is essential for interpretation of environmental stimuli and selection of a behavioral response." (de Kloet et al. 1999). Generally spoken, the activation of the MR seems to be associated with functions that in turn are connected to activation of the prefrontal cortex (PFC) or with the 'top-down' control of behavior (Miller and D'Esposito 2005). Numerous tests asses' different aspects of PFC function but the most prominent at least in the clinical field is the *Wisconsin Card Sorting Test*.

Whereas the ANT has been constructed after extensive investigation of the phenomenon of attention and has been designed following the state of the art in cognitive research in 2000, the *Wisconsin Card Sorting Test* (WCST) has been developed as a diagnostic tool to measure prefrontal functions as early as in 1948 (Berg 1948). The subjects are asked to sort a card following the color, shape or amount of presented symbols to a reference card without knowing the sorting rule or the occurrence of a rule shift. Rule acquisition can only be achieved by an accuracy feedback. Imaging studies have shown a contribution of ventro- and dorsolateral prefrontal cortex as well as the caudate nucleus to the action of shifting rules (Konishi et al. 1998; Monchi et al. 2001; Ko et al. 2008). The WCST measures a rather broad spectrum of cognitive abilities including visual processing, attention, working memory and executive control. The most informative measure is the amount of 'persevarative' errors indicating that the participant still sorts cards following the rule acquired before a shift took place (Barcelo and Knight 2002; Greve et al. 2005). In young healthy subjects only few errors occur and it has been shown that those are not randomly distributed. Rule shifts can either be extradimensional shifting from one category (e.g. sorting follows the rule "color" then shifts to "shape") or intradimensional shifting within one category (e.g. from "green" to "red"). Intradimensional shifts seem to be easier to detect than extradimensional shifts (Isaacs and Duncan 1962; Slamecka 1968) and they have been shown to activate distinct neural circuits pointing to a contribution of prefrontal areas mainly in extradimensional shifts (Konishi et al. 1998; Konishi et al. 1999; Konishi et al. 2002; Smith et al. 2004). Further it has been shown that working memory load and set shifting partly activate the same brain areas (Konishi et al. 1999) which is intuitively understandable because active rules have to be kept in mind to successfully shift rules.

The objective of the present study was to investigate the association of two SNPs in the MR gene and human cognitive function. The SNPs, MRI180V (rs5522) and MR-2G/C (rs2070951) have been described in section 2.5.4: "MR polymorphisms", and they were chosen because they had been shown to alter MR function *in vitro*. Further, they functionality in vivo as the different variants were associated with differences in challenged hypothalamus-pituitary-adrenal axis activity (DeRijk et al. 2006; van Leeuwen et al. 2010). Additionally very recently, the MRI180V has been shown to be related with reward learning under stressful and non-stressed conditions (Bogdan et al. 2010). For the assessment of cognitive function, the ANT and the WCST were chosen in order to measure executive PFC driven control processes. The ANT induces response conflict that has to be resolved by such control mechanisms using rather simple stimulus material. During the WCST, more complex cognitive processes take place and executive control is needed to successfully shift between rules.

3.2 Materials and Methods

3.2.1 Subjects

Initially, 156 students and co-workers of the University of Trier (mean age: 26 years, *SD*: 4.95) were recruited by the Email system of the University of Trier. In an initial telephone screening all subjects reported to have no chronic or acute disease, to be medication free (except for oral contraceptives) and to be non-smoker. To exclude the well documented influence of the menstrual cycle on cortisol measures all women used oral contraceptives. Females using a drospirenone containing oral contraceptive were not included due to the MR antagonistic effect of this progestine (Oelkers 2004). In a first step, participants came to the lab and were screened for ethnic decent by a short interview, asking where parents and grandparents were born. In the sample, there were 17 persons of non-German decent. Fifteen of them had parents or grandparents from Poland, Bulgaria or Romania, one person had an Afro-American and another one had an Indonesian grandfather. All participants gave written informed consent before the sampling of epithelial mouth cells. During the lab visit, buccal swabs were sampled from each volunteer for subsequent DNA isolation.

3.2.2 DNA treatment

DNA was isolated with the buccal cell Kit (Gentra Puregene Buccal Cell Kit, Quiagen, Hilden, Germany) according to the manufacturer's protocol. Samples were sent to the cooperation laboratory in Leiden and were genotyped for two single nucleotide polymorphisms (SNPs) in the mineralocorticoid MR gene *NR3C2*. Protocols for the genotyping of the two SNPs are described elsewhere (van Leeuwen et al. 2010). Due to a very low amount of isolated DNA genotyping failed in three subjects for MR -2G/C and in six subjects for MR I180V, respectively.

3.2.3 Genotyping Results

Because the main interest of the study was to characterize the function of MR polymorphisms in human cognitive functions with additional assessment of daytime and mildly stimulated cortisol levels, the genotyping was essential for the formation of experimental groups. The results are briefly reported here, as they were counted as a preparatory study for the main investigation.

In our sample we found a similar distribution of alleles for the two polymorphisms as described for other populations. The rarest allele of the two polymorphisms is the G allele of the MRI180V. In our sample we had an allele frequency of 11,33% for this allele and allele frequencies of both SNPs were comparable to other populations (DeRijk et al. 2006; Kuningas et al. 2007; van Leeuwen et al. 2010). For the MR-2G/C polymorphism both alleles were nearly equally distributed. The MR-2G/C C allele was present in ~53% and the G allele

in ~47% of all subjects genotyped. Consistent with the study by van Leeuwen et al. the MR I180V G allele never occurred in homozygous carriers of the MR-2G/C G allele. Additionally, there was only one participant homozygous for MRI180V G allele. Both SNPs are in Hardy Weinberg equilibrium (HWE) in the sample assessed in the present pre-study (MRI180V: χ_1^2 =0.0036; p=.952, MR-2G/C: χ_1^2=0.8819; p=.348).

MRI180V	MR-2G/C			
	CC	GC	GG	
GG	2 (1.33%)			2 (1.33%)
AG	19 (12.67%)	11 (7.33%)		30 (20%)
AA	24 (16%)	58 (38.67%)	36 (24%)	118 (78.67%)
	45 (30%)	69 (46%)	36 (24%)	150 (100%)

Table 3.1: Distribution of genotypes for MRI180V and MR-2G/C in all subjects entirely genotyped. For six subjects the amount of isolated DNA was not sufficient.

After genotyping for two polymorphisms in the MR gene (*NR3C2*: rs5522, rs2070951) a sample of 72 subjects (38 men, 34 women, mean age: 25.88, SD= 4.823) was chosen for further phenotyping. According to the genotype four about equally sized sex-matched comparison groups were composed. In the main study seven persons of partly non-German decent were included, four had a father or mother from Poland, three had one or two Romanian parents.

MRI180V	MR-2G/C				
	CC	GC	GG		
GG	1 (1.39%)			1 (1.39%)	180V (26,39%)
AG	10 (13.89%)	8 (11.11%)		18 (25%)	
AA	16 (22.22%) -2CC	18 (25%) -2G/C	19 (26.39%) -2GG	53 (73.61%)	
	27 (37.5%)	26 (36.11%)	19 (26.39%)	72 (100%)	

Table 3.2: Distribution of genotype groups – the 180V group consists of the MR genotypes 'highlighted' in grey; all homozygous carriers of the major A allele of MRI180V were divided in three further groups based on the MR-2G/C genotype: the group heterozygous for MR-2G/C will be referred to as -2GC, the homozygous groups are referred to as -2CC or -2GG respectively.

3.3 Experimental Procedure

Participants of the main study were invited for a visit in the psychophysiology laboratory at the University of Trier. Test sessions took place in the afternoon at around 2:15, 4:00 or 5:45 p.m. in order to avoid high levels of cortisol (see Chapter 5). Before preparation for the EEG measures, participants were asked to give written informed consent to attending the main study. The subjects were prepared for electroencephalogram (EEG) measurements (see Chapter 4) and were then left alone in the sound attenuated EEG chamber. The two tests (ANT and WCST) were presented in a computerized manner, providing the subjects with instructions how to proceed. Distance was adjusted to approximately 100 cm from a flat-panel LCD of 19 inch (48.26 cm – 24 x 42 cm) on which stimuli were presented.

3.3.1 ANT

The ANT is a combination of the Flanker Task (Eriksen and Schultz 1979) and different cue conditions preceding the flankers. Each trial begins with the presentation of a fixation cross which remains visible for 400 to 1600 ms (delay 1 – D1). It is followed by one of four cue condition cues that remains visible for 100 ms and is followed by a fixation cross which stays for 400 ms before the flankers appear. The reaction time is restricted to 1700 ms and contributes to the definition of length of the inter trial interval (ITI).
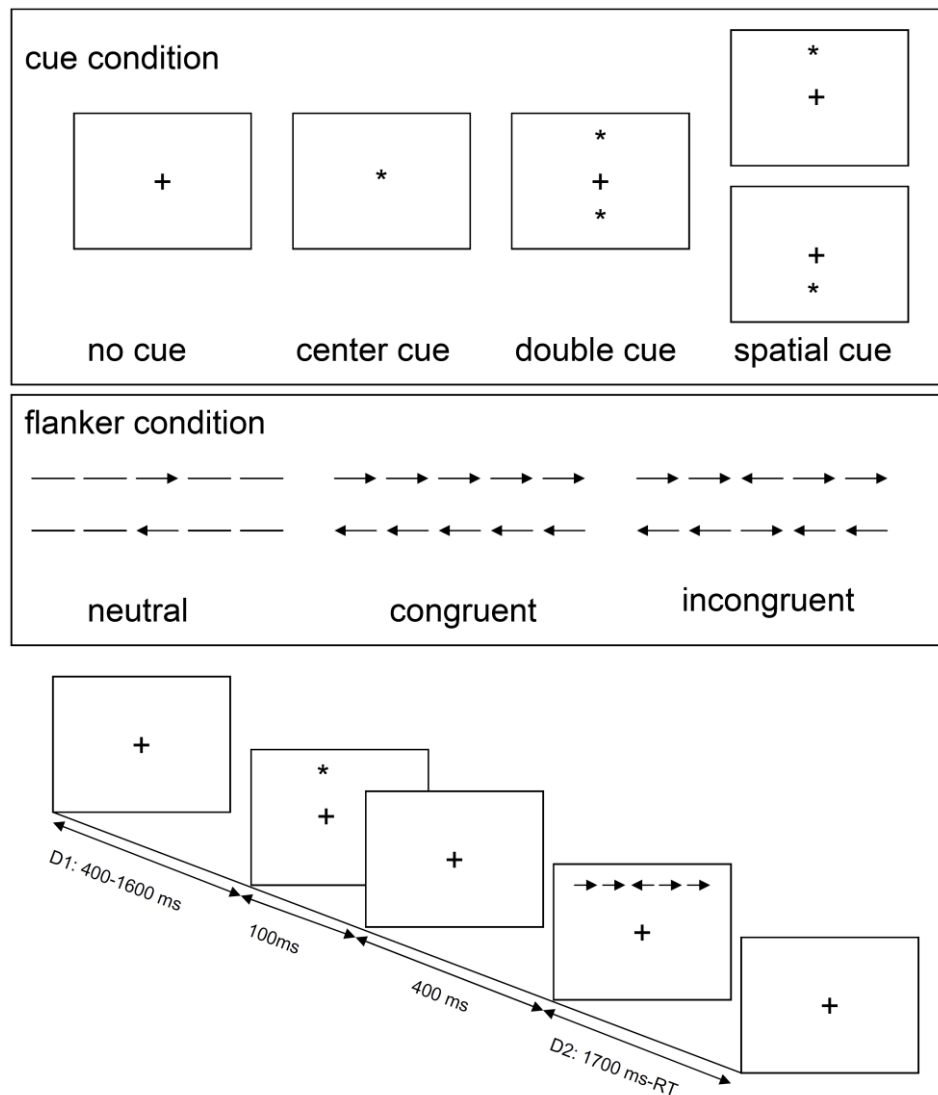
Figure 3.1: adapted from Fan et al. 2002.

The three factors of attention were measured by the subtraction of the reaction time of two different conditions. The alertness network was assessed by the difference of the no cue minus the double cue condition, the orienting network was calculated by center cue minus spatial cue trials and the conflict network is assessed by incongruent minus congruent flankers.

Participants were asked to indicate the direction of the middle arrow of the flanker line and they chose the direction on the left or right cursor tap of a customary computer keyboard with the fingers of their right hand. Participants resolved ten training trials before the ANT started. There were three blocks of 48 trials each; every possible combination of flankers (3), cues (4), direction of the middle arrow (2) and spatial position (2) was presented one time in each block in randomized order.

3.3.2 WCST (modified version)

Due to the methodological weaknesses mentioned above (see Introduction of this chapter) as mixing intradimensional and extradimensional shifts and a high working memory load it further did not seem appropriate to take the original form of the test. The version that was used for the study lacked one category of sorting ('amount') and allowed to distinguish between intradimensional and extradimensional shifts in a block-wise fashion (Watson et al. 2006). The participants were instructed to sort cards either by shape or by color and they were informed that rules will switch without further information. They were presented with three white cards on a black screen, each showing one of four symbols (square, circle, triangle or star) in one of four colors (red, yellow, blue or green). There was one card displayed in the top row and two cards in the bottom row. The subject was asked to sort the top card to one of the cards below by choosing the left or the right card with the fingers of their right hand on the cursor keys of a computer keyboard. Cards were presented until the participant reacted and feedback was presented for one second as written words in red for the "error" feedback and green for the "right" feedback. After feedback presentation, an ITI of one second followed before the next set of cards is presented.
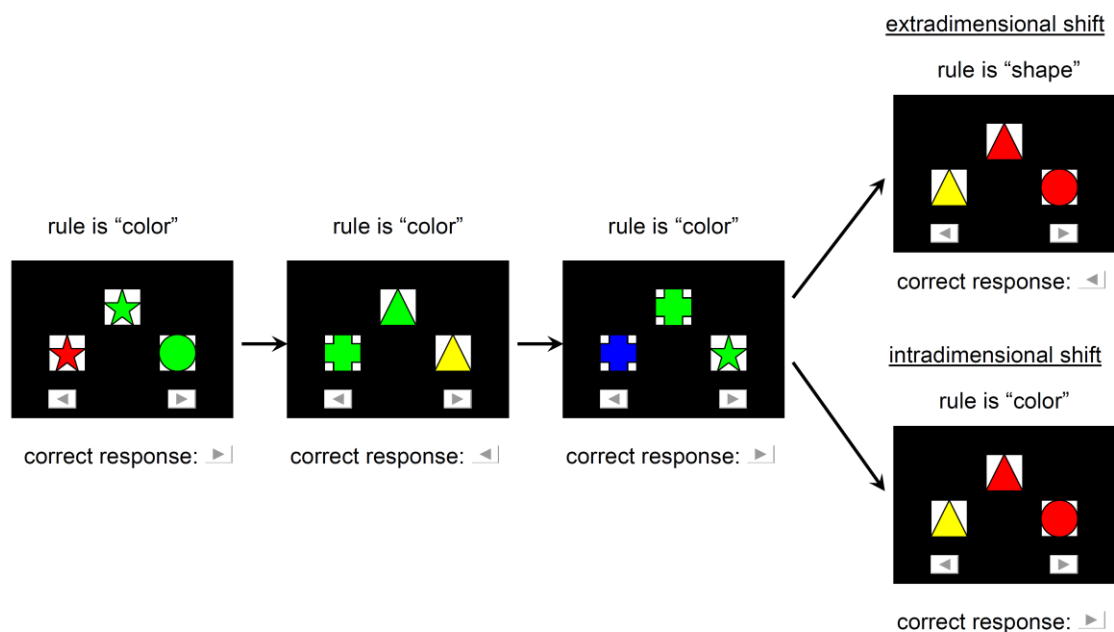
Figure 3.2: adapted from Watson, 2006 – example for the two possible kinds of shifts.

The WCST was structured in two blocks containing each 16 intradimensional and 16 extradimensional shifts so that for the whole WCST 32 intradimensional and 32 extradimensional shifts took place (for an example of intra- vs. extradimensional shift see Figure 3.3). In order to avoid anticipation of upcoming shifts, rules lasted for either five or six trials (pseudo-randomized) before the next set shift.

3.4 Statistical Analysis

In a first step, an analysis of outliers was carried out for both tests. For the ANT, reaction times differing more then 2 standard deviations from the general mean were excluded from further analysis. For the WCST, variations of reaction times were very high (as they were not limited like in the ANT) and an all over cut-off for reaction times of 3500 ms was set; i.e. all responses that occurred later then 3500 ms were excluded from the analysis.

We carried out an ANOVA for repeated measures to analyze the ANT. The repeated measures factors were: network (alertness, conflict, orienting), and condition (no cue, double cue, incongruent flanker, congruent flanker, center cue and spatial cue). Genotype was integrated as between subject factor and models were calculated for both, reaction times and error rates of the answers.

For the WCST we tested the first three trials of intradimensional and extradimensional shifts in two models – one for reaction times and one for error rates. Here, the shift type (intradimensional/extradimensional) and the number of trial (one, two, and three) were integrated as repeated measures. In both models the genotype was integrated as between-subject factor to look for possible influences of MR polymorphisms on behavioral measures. The main interest was to reveal main effects for conditions of both tests to check if they require different cognitive demands which were reflected in reaction times and accuracy of the answers. For WCST we additionally looked for an interaction of shift type and number of trial as it seems probable that first trials at least for extradimensional shifts are more difficult to resolve than the following and that the picture is different for first intradimensional shift trials.

In the case of significant interactions that contained the genotype, the *Dunn's Procedure of Multiple Comparisons* was carried out. The Dunn's procedure is less conservative than the Bonferroni adjustment, because it takes into account the number of comparisons that have been made compared to all possible comparisons for the Bonferroni adjustment. Critical values for comparisons are provided by tables for p values of 5% or 1%, taking into account the number of comparisons made and the error degrees of freedom of the respective interaction. This critical value then is related to the error mean sum of squares, and an index for the number of subjects (multiplied with the level number of factors not integrated in the interaction). The resulting values indicate the critical difference for single comparisons on either a 5% or 1% level.

After visual inspection of individual cortisol profiles during the EEG session, some subjects (n=19) were identified that responded to the test situation with rise in salivary cortisol of more than 2nmol/l. These subjects were labeled as 'responders' and we analyzed additional ANOVAs for a possible effect of being responder or non-responder on reaction times or accuracy in the ANT or WCST.

3.5 Results

3.5.1 ANT

For both reaction time and error rates we found significant main effects for network, difference factor and their interaction. In general, differences in reaction times have larger effect sizes than differences in accuracy. The main effect of network (reaction times: $F_{2/136}$=360.566; p< .0001 HF; η_p^2= .841) indicated a higher reaction time in the orienting network, followed by the executive control and the alertness network on a descriptive level. The main effect of difference factor ($F_{1/68}$=838.715; p< .0001; η_p^2=0.925) showed that averaged over all networks there was one condition that provoked higher reaction times. The interaction between network and difference factor ($F_{2/136}$=43.17; p<.0001; η_p^2= .388) revealed higher reaction time differences between the cue conditions of the orienting network and the flanker conditions in the conflict network compared with reaction times in the alertness network. For a general overview of reaction times see Figure 3.4. It shows the main effects for cue conditions and flankers and visualize the mean reaction time differences of the four cue conditions within the three flanker conditions and vice versa. Table 3.3 gives the same information as Figure 3.4 plus the standard deviations of the respective cue/flanker combinations and reaction times and standard deviations for the flanker conditions, averaged over all cue conditions as well as for the cue conditions, averaged over all flanker conditions.
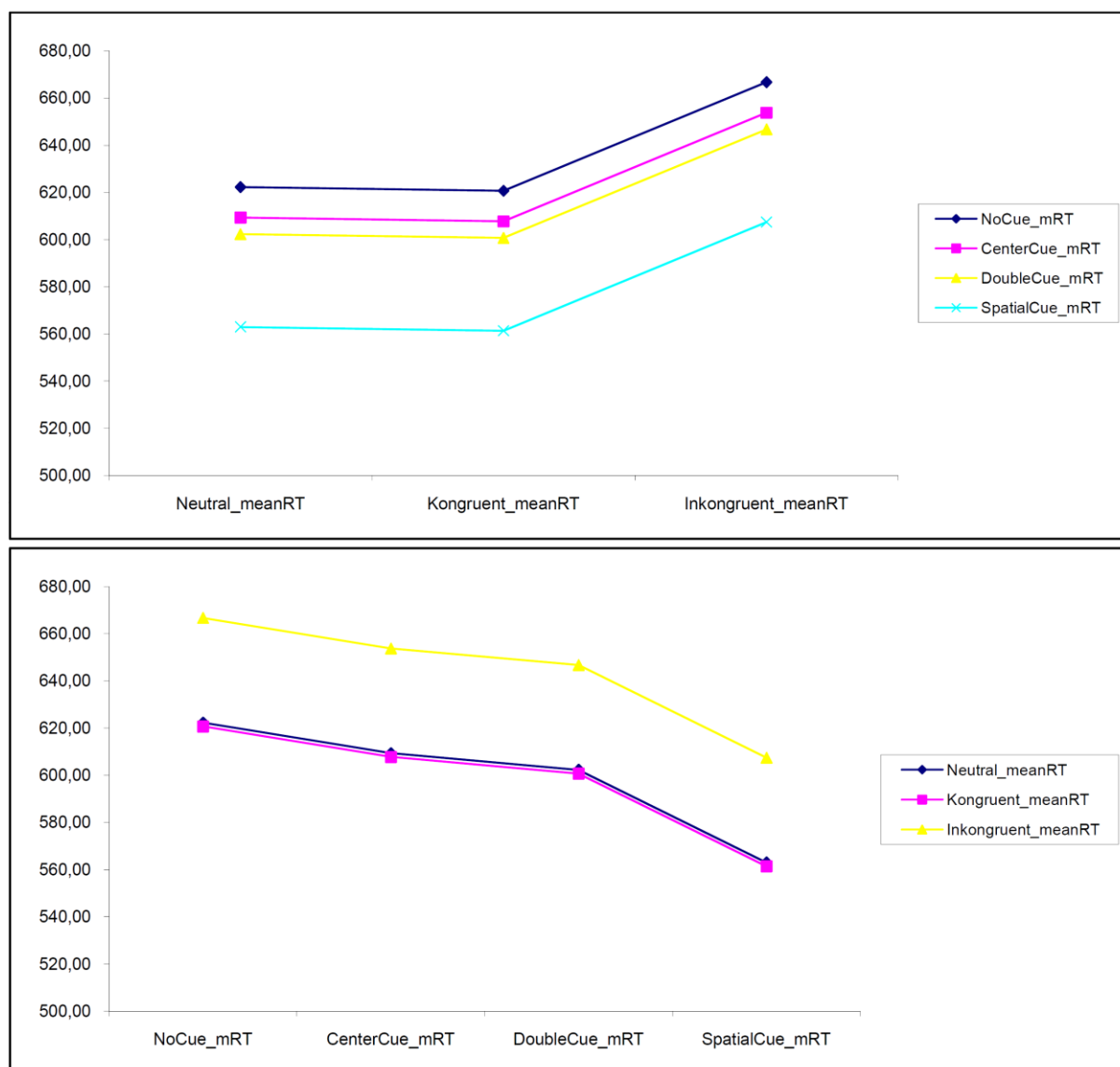
Figure 3.3: Mean reaction times for cue conditions in different flanker conditions and vice versa. Note that for the sake of clearness no error bars were included.

	NoCue	CenterCue	DoubleCue	SpatialCue	
Neutral	622.3308 (57.07)	609.3740 (57.75)	602.3203 (57.01)	563.0141 (62.62)	599.2598 (58.61)
Congruent	620.7169 (60.46)	607.7601 (61.14)	600.7065 (60.40)	561.4002 (66.01)	597.6459 (62.00)
Incongruent	666.7747 (72.54)	653.8179 (73.22)	646.7642 (72.48)	607.458 (62.62)	643.7037 (70.22)
	636.6075 (63.36)	623.6507 (64.04)	616.5970 (63.30)	577.2908 (63.75)	

Table 3.3: Mean reaction times and (standard deviations) for all condition combinations.

To check if the three networks could be considered as independent, the intercorrelations for the reaction time differences characterizing the networks were calculated (see Table 3.4). There were no significant correlations between reaction time differences in the three attention networks in our sample. Nevertheless the statistically significant interaction between network and difference factor indicated that the networks show interdependence. In every network, there is one difference factor that is suggested to be the one to which it is more difficult to react. In the case of the alertness and the orienting network these stimuli consist of one of the cue conditions (no cue or, respectively, center cue). As the reaction time depends on the reaction to the flanker row, at least the conflict network will always be related to either the alertness or the orienting network, as the corresponding cue conditions precede the flankers that count for the conflict network. Still, the non-significant intercorrelations between the network indices suggested that the reaction time differences between two conditions, which together built up one network, were not dependent one from the other.

		conflict	alertness	orienting
conflict	Pearson's r^2	**1.00**	**-0.03**	**-0.04**
	significance (two-tailed)		0.82	0.73
alertness	Pearson's r^2		**1.00**	**0.05**
	significance (two-tailed)			0.67
orienting	Pearson's r^2			**1.00**
	significance (two-tailed)			

Table 3.4: Intercorrelations of the attention networks in our sample and two-tailed significances of correlations.

For error rates, the main effect of network ($F_{2/136}=11.854$; $p< .0001$ HF; $\eta_p^2=.148$) indicated higher error rates in the executive control/conflict network and lowest error rates for the orienting network. The main effect of difference factor ($F_{1/68}=33.292$; $p<.0001$. $\eta_p^2=.329$) revealed that averaged over all networks, one of the difference factors was associated with higher error rates. The interaction between network and difference factor ($F_{2/136}=17.329$;

p<.0001 HF; η_p^2=.203) showed that the difference between error rates of the two conditions is highest in the executive control network, with higher error rates for incongruent flankers. The orienting network was characterized by the smallest error rate difference, with the exception that in the spatial cue condition, which would normally be described as the 'easier' condition, error rates are higher than in the center cue condition.

For error rates, we found a significant interaction between network and genotype group ($F_{6/136}$=2.482; p=.032 HF; η_p^2=.099).The -2G/C subjects committed fewer errors in the alertness network than in the orienting network. Regarding the two conditions of the orienting network, this is mainly due to a higher error rate for the spatial cue condition then in the center cue condition. For the other three genetic groups the error rates in the orienting network are smaller then in the alertness- or the conflict network. On a descriptive level the -2G/C show the lowest overall error rates, followed by the -2GG group which still makes less faults then MR I180V G carriers and the MR-2 C homozygotes. In the Dunn's post hoc test only the group differences between the 180V group and the –2CC group were not significant in any of the networks, as shown in Figure 3.5. All other group differences turned out to be significant at a 1% level in the conflict and alertness network. In orienting the 180V group had significantly higher error rates then -2G/C and -2GG and -2CC subjects performed significantly worse than the -2GC group.
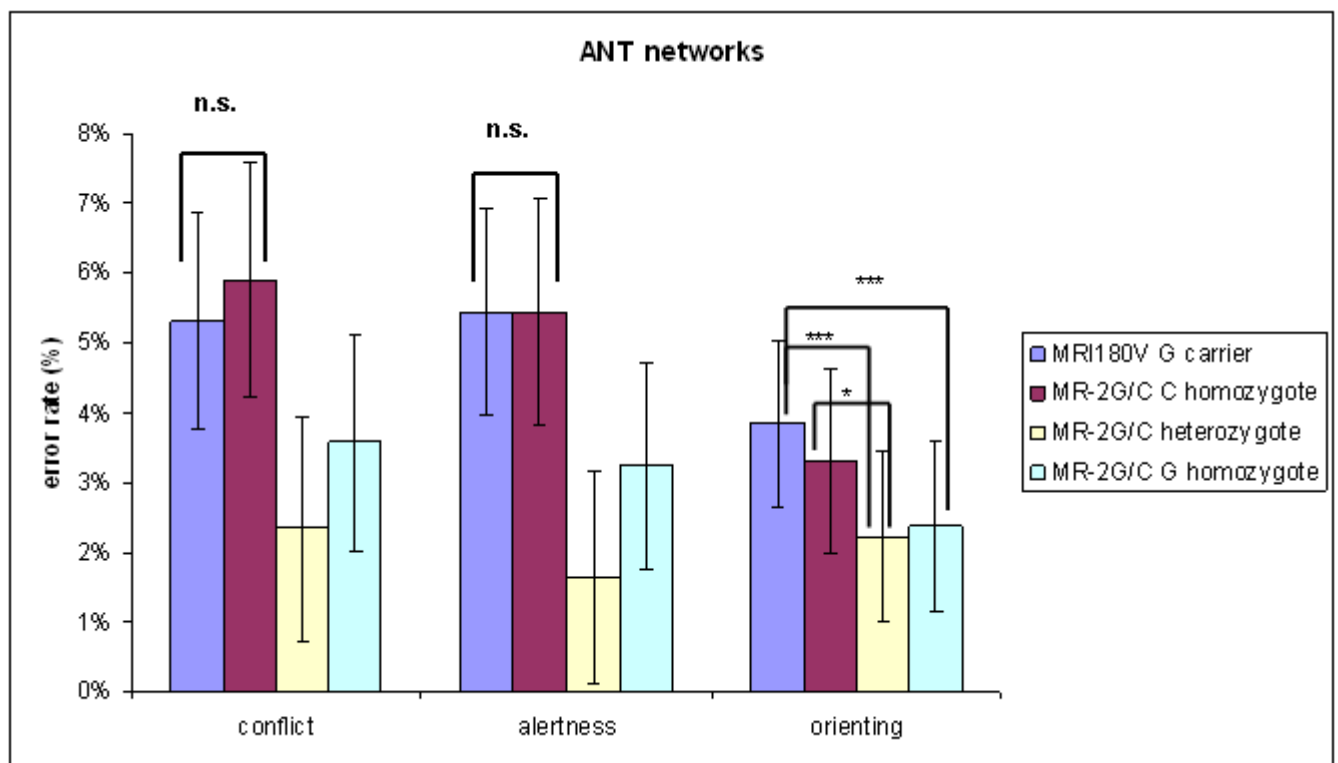
Figure 3.4: Error rates for the three ANT networks, in the conflict and alertness network all group differences apart from the comparisons of 180V and -2CC were

significant. Error bars indicate the standard error. (***: absolute difference exceeding the critical difference for p=.01 following Dunn's Procedure of Multiple Comparisons.)

3.5.2 WCST

There were significant main effects for both the type of shift ($F_{1/68}$=52.903; p<.0001; η_p^2= .438) indicating higher reaction times for extradimensional shifts than for intradimensional shifts. A main effect of trial number ($F_{2/136}$=11.983; p<.0001 HF; η_p^2=.15) showed that reacting to the second trial after rule shifting took the longest reaction times. Further there was a significant interaction of shift type and trial number ($F_{2/136}$=64.052; p<.0001 HF; η_p^2=.485) revealing that the second trial of extradimensional shifts provoked the highest reaction times.

In the model calculated for accuracy of the answers there was a significant main effect for shift type ($F_{1/68}$=1954.132; p<.0001; η_p^2=.966) which showed that after an extradimensional shift, subjects committed more errors than after an intradimensional shift. The main effect of trial number ($F_{2/136}$=4370.819; p<.0001 HF; η_p^2=.985) indicated the most errors in first trials. Finally, the interaction of shift type and trial number ($F_{2/136}$=1351.819; p<.0001 HF; η_p^2=.952) confirmed that in first trials after an extradimensional shift, the most errors were made.

The interaction between trial number and genotype failed to be significant like genotype as between-subject factor and the three way interaction of shift, trial, and genotype.

For accuracy of the answers there was a trend towards significance in the interaction of shift and genotype ($F_{1/68}$=2.191; p=.097; η_p^2=.088) indicating differences between genotype groups for intradimensional shifts. In our sample the -2G/C and -2CC group showed the highest error rate but whereas the -2CC subjects further made the most faults than the other groups, the -2C/G group showed the steepest learning curve. In the third trial, the -2GC group caught up with the -2GG group that from the first trial on showed the lowest error rate. The 180V group showed a particularity: while the subjects made the right choice at a high percentage in the first intradimensional shift trials and improved to the second one, they decreased (very) slightly in their performance in the third trial. As shown in Figure 3.6, on a descriptive leves these group differences were rather small.

intradimensional shifts

Figure 3.5: Percentage of correct answers in the first three trials of an intradimensional shift in the four genotype groups. Note that the y-axis is labeled in percentage of accuracy (not in error rates as for the ANT). Error bars indicate the standard error.

3.5.3 Responder/non-Responder analysis

There were no significant effects of being responder to the test session on reaction times or accuracy scores of the first three trials of intradimensional and extradimensional shifts in WCST. For ANT there was no effect of being responder or not on reaction times but for accuracy of answers a trend towards a significant interaction of responder and difference factor ($F_{1/66}$=3.462; p=.067; η_p^2= .05) was found. This effect suggested that responders made fewer errors in the conditions that were considered as the more difficult conditions of a network (no cue, center cue or incongruent flankers) but the effect size for this interaction was low. Because of the low effect size, we didn't integrate responder/non-responder as covariate in the models analyzed for genotype. Nevertheless this interaction revealed interesting implications for the impact of cortisol secretion on attention networks, which will be discussed below.

3.6 Discussion

The analysis of response differences between the tests conditions used for the phenotyping of our sample revealed in general that the manipulation of different cognitive demands had been successful. For the ANT, reaction times were shortest for the spatial cue condition followed by the conditions double cue, center cue and no cue. Reaction times for neutral and

congruent flankers did not markedly differ but both were significantly shorter than those for incongruent flankers. Accuracy is most important for the flanker conditions as subjects react to indicate the direction to which the middle arrow points. Accuracy was not substantially different between neutral (mean: 1.44%, *SD*: 0.0488) and congruent flankers (mean incorrect answers: 1.17%, *SD*: 0.0534) but increased when the middle arrow pointed to the opposite direction than the surrounding flankers (mean: 7.32%, *SD*: 0.106). The interaction between network and difference factor cues or flankers indicated that the reaction to flankers could be modulated by the different warning cue conditions. In all cue conditions it took the subjects longer to react to incongruent flankers but when a spatial cue appeared, subjects reacted faster to all of the three flanker conditions comparable to healthy controls in the obesity study mentioned above (Beutel et al. 2006). In another healthy population tested to assess the "efficiency and independence of attentional networks" a similar interaction has been found. In difference to our sample participants didn't only show faster reaction times when presented with a spatial cue but reacted more slowly to flankers after the presentation of a center cue (Fan et al. 2002).

There was a significant interaction of network and genotype for error rates in our sample suggesting that the C allele of the MR-2G/C polymorphism and the minor allele of MRI180V are associated with an impairment of the ability to react correctly to a flanker task. This effect was modulated by the warning cue given before the flankers appear. While these group differences existed for accuracy of the answers, the MR genotype was not related to reaction times at all. The effect size of the interaction is comparable with the association Reuter et al. found for higher error rates in the execution network in homozygous carriers of the minor allele of a polymorphism in *TPH2* (rs4570625). In our sample we found significant group differences in all networks, which until to date has not been reported. In most studies only the execution network shows associations with genetic polymorphisms (Fossella et al. 2002; Fan et al. 2003; Reuter et al. 2007) or (psycho-) pathology (Beutel et al. 2006; Gooding et al. 2006; Leskin and White 2007).

For the WCST it can be seen in the results of the behavioral data that extradimensional and intradimensional shifts have different degrees of difficulty. In fact, intradimensional shifts are detected as shift much easier than extradimensional shifts. While in the first trial after the change of the sorting rule from one category to the other the sorting was incorrect in 94% of the trials (*SD*.: 0.1382) the first trial after the change between members of the same category is correctly sorted in 91% (*SD*.: 0.0881) of all cases. That means that intradimensional shifts are easier to identify than rule shifts. It is simply not possible to apply the previous rule (e.g. red) when it changes to another proxy of the same category (e.g. green). There are two ways to respond to this kind of shift: either the subject stays within the same category, which can be considered a "conservative" choice, which results in a correct decision when an

intradimensional shift occurs. Alternatively, the subject detects that there is a shift and directly performs an extradimensional shift and thus sorts by a different category.

The -2GG group showed the lowest error rate from the beginning. That might indicate a general tendency to sort cards following a 'conservative' strategy, which in the case of intradimensional shifts is very effective. The 180V group showed a comparably low error rate as the -2GG group but did not reach the same accuracy of answers until the third trial after an intradimensional shift. The slight decrease in accuracy from second to the third shift should be interpreted very cautiously as it only accounted for not more than 0.005%. Nonetheless, an impaired ability to perform an intradimensional shift could be related to deficits in working memory. Further it could be argued that impaired intradimensional set shifting could be associated with impaired reward learning (Bogdan et al. 2010) because the 'conservative' shift strategy is rewarded more frequently. But as already mentioned both argumentations would be very speculative. The -2CC group committed the most errors in the first three trials after an intradimensional shift but steadily improved in performance. Finally, the -2G/C group began with a comparably high error rate and could thus be characterized by a tendency to misinterpret intradimensional shifts as extradimensional. However, they improved quickly in accuracy of the answers and consequently displayed the steepest learning curve.

The detected association between intradimensional shifts and MR genotype is remarkable but it is difficult to integrate in the existing literature on MR function in human cognition. Otte et al. (2007) showed effects of pharmacological blockade of MR in tests that are dissimilar to the WCST paradigm we used in this study. In their experiment they found effects of MR function on performance in the *d2' test* (Brickenkamp 1978), the *Rey-Ostierrieth and Taylor Complex Figure Test* (Osterrieth 1944) and Part B of the *Trail Making Test* (Reitan 1992). In the WCST paradigm the task consists of the detection of rule changes and the interpretation of feedback to react appropriately in terms of following a learned rule. It thus requires involvement of novelty detection, 'habituation', and searching strategies by interpretation of feedback – behaviors that have been shown to be affected by MR function in rodents (Oitzl and de Kloet 1992; Oitzl et al. 1997; Berger et al. 2006; Lai et al. 2007; Rozeboom et al. 2007).

In the ANT as well as in the WCST the genotype groups differed in the amount of correct responses but whereas the -2GG and the 180V group had higher error rates in the ANT, they showed more correct answers in at least the first trial after an intradimensional shift in the WCST. This is surely due to the fact that the ANT and the WCST measure different cognitive operations that have been shown to activate only partly overlapping brain regions. While the ANT is a simple speed/accuracy task, the WCST requires informational processing of feedback as well as working memory performance. There is no feedback to the reaction in

the flanker tasks of the ANT whereas in the WCST the feedback is essential to resolve the task of set switching. In general, our results point in a similar direction as those of Otte et al. showing an impact of MR to selective attention and more complex cognitive operations including set shifting (as in part B of the *Trail Making Test*).

Responder – participants that reacted to the test situation with a rise in cortisol levels of at least 2 nmol – didn't react differently to WCST than non-responders. However, in the ANT, responders committed more faults than non-responders in the orienting network on a descriptive level. This difference is characterized by a bigger difference in the error rates between center and spatial cues in responders than in non-responders. Additionally, responders showed a relatively bigger difference in error rates between the two flanker conditions in the conflict network, compared to non-responders. For congruent flankers they made less incorrect decisions than participants who didn't show a cortisol reaction to the test session but they made more faults under the incongruent flanker condition. We didn't carry out a post hoc test because the comparison groups had very unequal sizes (19 responders vs. 49 non-responders). We neither integrated this dichotomous variable as a covariate into the GLMs calculated for the impact of genotype because responders were equally distributed among genetic groups (see chapter: cortisol) and the effect size was considerably low. Nevertheless it is interesting that the largest difference was found in the orienting network, pointing at an involvement of cortisol in visuospatial orientation as it has been investigated in rodents and humans (Schwabe et al. 2007; Schwabe et al. 2008; Schwabe et al. 2009; Schwabe et al.).

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CHAPTER 4

Mineralocorticoid Receptor polymorphisms are related to cortical activity during motor responses in ANT and extra-dimensional set shifting in WCST

Chapter 4: Mineralcorticoid Receptor Polymorphisms are related to cortical activity during motor responses in ANT and extradimensional set shifting in WCST

Background: The Mineralocorticoid Receptor (MR) is expressed in brain regions that are important for a broad range of cognitive functions and is implicated in mechanisms of neuronal excitability. Furthermore, the MR has been shown to influence attention, visuo-spatial memory, set shifting, and reward related behavioural adaption.

Methods: 72 subjects, previously genotyped for two single nucleotide polymorphisms (SNPs) in the MR gene were invited to perform the *Attention Network Task* (ANT) and a modified version of the *Wisconsin Card Sorting Test* (WCST) while their electroencephalogram (EEG) was recorded. Event-related potentials (ERPs) were calculated for different test conditions and analyzed for the influence of MR polymorphisms on changes of cortical activity during task performance.

Results: A significant interaction between genotype, electrode position, laterality, and condition was found for the ANT alertness network. Furthermore, we found a marginally significant main effect of genotype in the ANT conflict network. In both networks, activity differences occurred at the time of the motor response. In extradimensional shifts of the WCST a tendency for a significant interaction of position and genotype as well as a marginally significant interaction between time window, condition, and genotype was found.

Conclusions: MR polymorphisms are associated with cortical activity in attention and set shifting. During ANT, cortical activity differs between genotype groups at the time of reaction in the alertness and executive function networks. For the WCST, differences between genotype groups are related with feedback reception at the time of set shifting.

4.1 Introduction

The HPA is regulated by suprahypothalamic structures that are important for different cognitive processes. In those structures, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are co-expressed and the MR/GR ratio is heavily region- and tissue dependent, as has been described in Chapter 2. In human research, the link between cortisol and cognition has focused for a long time on functions of the hippocampus (HC) as a prominent GR expressing brain region. With growing knowledge about receptor distribution in the human brain, the research interest has shifted to the prefrontal cortex (PFC) as MR and, above all, GR expressing brain structure. Chronic administration of hydrocortisone has been shown to impair search strategy in a spatial working memory task and was associated with shorter reaction times (RTs) in a pattern and spatial recognition task (Young et al. 1999). Presumably, these results point to an involvement of the PFC because performance in the same tasks have been shown to be impaired in patients with frontal lobe excisions, but not in neurosurgical cases after unilateral amygdala-hippocampectomy (Owen et al. 1995; Owen et al. 1996). As a cortical structure, the PFC is ascertainable by electroencephalograms (EEGs) and this technique has been used to confirm its role in cortisol sensitive memory processes as well as other, memory unrelated cognitive tasks. For example, chronic administration (2 x 20mg per day for one week) of cortisol has been shown to impair episodic memory as measured by recognition accuracy. Additionally, the differences between the experimental and the control (placebo) group were accompanied by differences in event related potentials (ERPs). Correct rejections (vs. correctly recognized items) thereby were characterized by an enhanced positivity of waveforms on frontal electrodes in the cortisol group (McAllister-Williams and Rugg 2002). Thus nowadays, discussion of GC mediated effects on memory encompasses the functioning of neuronal networks and often focuses on the function of the PFC. Further, a single dose of 100 mg cortisol increases error rates in a Stroop task, which is accompanied by an enhancement of error related positivity (200 to 500 ms after the response) on a frontocentral electrode, leaving ERPs for correct answers unaffected (Hsu et al. 2003). In the latter study, episodic memory was assessed with the same task as in the study carried out by McAllister-Williams and Rugg, but it was unaffected by acute cortisol administration in respect to recognition and error rates as well as electrophysiological measurements. The Stroop task does not assess memory processes but is an instrument to measure executive functions. Executive functions have been characterized as functions that are needed in situations that involve planning or decision making, error correction or troubleshooting, where responses are not well-rehearsed or situations which contain novel sequences of action. They are important in dangerous or technically difficult situations and in such situations that require overcoming habitual responses or resisting temptation (Norman and Shallice 1986). The definition already implies that executive functions are a

heterogeneous class of cognitive operations, involving many distinguishable processes or functions i.e. detection of task novelty, perceptual difficulty of given stimuli, number of elements in working memory, and, most prominently: resolving response conflict. All of these functions, in turn, can be ascribed to be related with the activity of different subregions of the PFC (Duncan 2001).

An example for a stress related behavior that activates frontolimbic motivational networks integrating the PFC is the initiation or inhibition of an approach or avoidance reaction to social cues. The approach-avoidance (AA) task (Rotteveel and Phaf 2004) uses a well established protocol to induce affect congruent vs. incongruent arm movements in reaction to either angry or happy faces. In this task, a consistent effect reveals shorter reaction times for affect congruent movements. This congruency effect has been shown to be enhanced in a group of highly *Trier Social Stress Test* (TSST) responsive subjects when carried out before the TSST and is diminished during TSST whereas the low responders showed the opposite pattern of the congruency effect (Roelofs et al. 2005). Further, administration of cortisol has shown to alter ERP waveforms recorded during the avoiding arm movement on frontal electrodes (van Peer et al. 2007). In both studies cited here, trait avoidance was assessed by the *Behavior Inhibition Scale* (BIS) (Carver and White 1994) and was shown to mediate the effects of cortisol on approach-avoidance behavior. In both studies, only in those subgroups with BIS scores, the found effects of cortisol were significant. Subjects suffering from social phobia seem to be most sensitive for the effect of psychosocial stress on the inhibition of an avoidance reaction to angry faces (Roelofs et al. 2009). After administration of a single dose of cortisol nonetheless, this effect does not appear in reaction times, whereas an early positive amplitude in the corresponding ERPs has been shown to be enhanced only in those social phobic participants that had the most severe symptoms (van Peer et al. 2009). Taken together, the results of Roelofs and colleagues could speak for context specificity of cortisol effects in the processing of emotional stimuli that are apparent already before memory processes take place.

All the results reported here, aimed to investigate effects of high levels of endogenous and exogenous cortisol on cortical activity and consequently it could be argued that all those effects are GR mediated. On the other hand, the neuroendocrine systems underlying 'stress', 'allostatic load', or 'homeostasis' are highly plastic and vary continuously in readiness to react to external cues (Chrousos 2007), they can change in sensitivity over the lifetime (Lupien et al. 2009) and react in a situation specific way, thereby reflecting individual dispositions (Spijker and van Rossum 2009) and/or experiences (McEwen and Gianaros 2010). To investigate MR mediated functions, experiments should be carried out at times of relatively low levels of circulating cortisol in order to avoid GR mediated effects. Experimental group designs can be achieved by the comparison of MR pharmacological blockade vs.

placebo or by the construction of groups based on the MR genotype. Both approaches have been used in one study each until now and an association between MR functioning and several cognitive tasks have been described. As already mentioned in Chapter 2, administration of an MR antagonist lead to impaired selective attention, as assessed by the d2' test (Brickenkamp 1978), visuospatial memory, measured by the accuracy of retaining complex figures (Osterrieth 1944) and set shifting or cognitive flexibility, assessed by the second part of the trail making test (Reitan 1992; Otte et al. 2007). A very recent study reports of an association of a polymorphism in the MR gene (MRI180V, rs5522) and reward learning as well as reward learning under stress. Carriers of the rare G allele of this polymorphism showed enhanced reward learning under non-stressed conditions compared to homozygous carriers of the common A allele. Further, stress induced deficits in reward learning were largest in MRI180V G carriers (Bogdan et al. 2010).

The aim of the present study was to investigate the association between two single nucleotide polymorphisms (SNPs) of the MR gene and cognitive functions. Cognitive tests that were used for the study were the *Attention Network Task* (ANT) and a modified version of the *Wisconsin Card Sorting Test* (WCST). The ANT was used because it is a paradigm of minimal complexity of the stimulus material as well as minimal task performance demands. It measures three distinct networks that contribute to the ability to activate and direct attention as well as resolving response conflict. Attention processes have repeatedly been shown to be mediated by cortisol (Fehm-Wolfsdorf et al. 1993; Skosnik et al. 2000; van Honk et al. 2000) and particularly, involvement of the MR was shown for the d2' test (Otte et al. 2007). Additionally, subjects attended to a modified version of the WCST as a measure of cognitive flexibility as a relative complex cognitive task. The WCST was chosen to assess set shifting abilities as MR blockade have been shown to impair those. Further, the aspect of feedback guided rule detection seemed very interesting as it integrates some kind of reward oriented learning.

The SNPs we investigated are described in detail in Chapter 3 as well as the cognitive tests that were used. We chose the method of ERP assessment because of the very high time resolution of the method that allows detecting rapid changes in cortical activity. The MR might influence very fast cognitive processes, considering its function for synaptic excitability (Karst et al. 2005; Olijslagers et al. 2008). Because this is the first study assessing the impact of MR polymorphisms on cortical activity deflections we did not choose ERP components a priori but used an exploratory approach (see below under "Data processing"). An additional reason for the exploratory approach is the lack of studies using the same tasks to assess the effect of cortisol on EEG measures.

4.2 Subjects

72 right-handed participants (38 man, 34 women, mean age: 25.88, *SD*= 4.823) of the previously genotyped population were invited to the psychophysiology lab of the Trier University to take part in the main study. Experimental groups were based on the participants' MR genotype (for details see Chapter 3). The MR180V group (10 men, 9 women, mean age: 26.33, *SD*= 5.65) contained all carriers of the rare G allele of MRI180V and was heterogeneous for MR-2G/C. The other three groups were homozygous for the A allele of MRI180V and differed concerning the biallelic occurrence of MR-2G/C. The homozygous carriers of the C allele will be referred to as MR-2CC (10 men, 6 women, mean age: 23.6, *SD*= 2.85), heterozygous subjects as MR-2GC (9 men, 9 women, mean age: 27.56, *SD*= 6.25) and G homozygous participants as MR-2GG (10 women, 9 men, mean age: 25.95, *SD*= 3.03) All subjects had normal or corrected-to-normal vision.

4.3 Experimental Procedure

The EEG test sessions took place in the afternoon between 2:15 and 6:00 p.m. in order to avoid high cortisol levels (Schreiber et al. 2006) that could lead to confounding effects of GR function on test performance. After reception, the participants were comfortably seated in a dimly lit sound attenuated chamber. They were asked to give written informed consent to participate in the main study. Distance was adjusted to approximately 100 cm from a 19 inch (48.26 cm – 24 x 42 cm) flat-panel LCD on which stimuli were presented. Subjects responded to the flanker direction for ANT or the position of the target card for WCST by pressing the left and right cursor taps of a customary keyboard by the fingers of the right hand.

4.3.1 ANT

The ANT has been described in detail in Chapter 3 and was carried out to distinguish performance in very well described neuronal networks contributing to attention. We are not aware of EEG studies assessing performance of the ANT and heritance of the different networks has been described in the aforementioned chapter. The aim of the investigation was to detect differences of the activity between genetic groups in those cortical areas that contribute to test performance and are part of MR expressing networks.
Stimuli were presented as shown in Figure 4.1 and ERPs were time locked online for the onset of cue presentation, flanker presentation (additional: position and direction of middle arrow), and no, correct or false responses. The program automatically registers coded 'starting points' for ERP averaging conditions within one subject and averages over all subjects (grand average). These averages were registered and can subsequently be exported for further statistical analysis. These ERP starting points will further be referred to

as 'triggers'. The whole line of flankers had a length of 5.8 cm of which each arrow had a length of 1 cm with 0.2 cm of space between the arrows. The flanker row was presented either above or below the middle of the screen, each arrow subtended a visual angle of 0.57° and the whole flanker row subtended a visual angle of 3.32°.
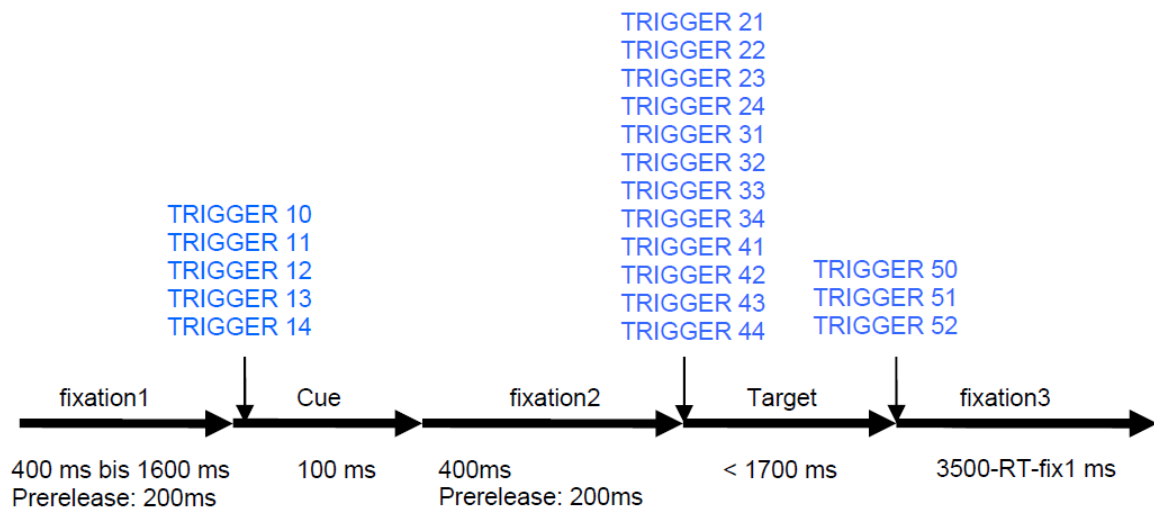
Figure 4.1: time line and triggers set for EEG recordings of the ANT - trigger 1X: cue condition (no: 0, center: 1, double: 2, spatial up: 3, spatial down: 4), trigger 2X: neutral flanker (up left: 1, up right: 2, down left: 3, down right: 4), trigger 3X: congruent flanker, trigger 4X: incongruent flanker, trigger 5X: response (no: 0, correct: 1, false: 2).

4.3.2 WCST

The WCST is a test that has been used for clinical diagnostic of prefrontal dysfunction since 1948 (Berg 1948) but has been validated by electrophysiological and neuroimaging techniques much later. There is converging evidence for an activation of prefrontal areas (dorsolateral, ventromedial and orbitofrontal PFC) as well as many other brain regions, such as inferior parietal cortex, temporo-parietal association cortex, temporo-occipital cortex, temporal pole, visual cortices during task performance in healthy participants. However, the results of previous studies are less consistent regarding task-related changes in the activity the thalamus, basal ganglia, the parahippocampal gyrus, and HC. Therefore, WCST can be regarded as a measure for higher-order cognitive functions characterized by distributed neural networks. Which areas are found to be activated heavily depends on the experimental design (Barcelo 2001). So far, one study reports a moderate but significant influence of heritability on some WCST indices (number and percentage of errors, the number of perseverative responses, and the number and percent of perseverative errors) (Anokhin et al. 2003). Furthermore, Egeland and colleagues (Egeland et al. 2005; McCormick et al. 2007) found influences sex-dependent influences of basal cortisol on WCST performance in

healthy and depressive individuals. This may point to a contribution of HPA axis activity on PFC dependent cognitive flexibility in humans.

The WCST version used in the present study differed from the original task in that it lacks one of the original sorting categories (amount) and allows unambiguous sorting of cards. As there can be only one efficient fault in this paradigm, the moment of set shifting can be "localized" properly to take place after the last false trial in one rule block and will relate on the information given by the feedback. We concentrated on analyzing extradimensional shifts for two reasons: first, it has been shown that in this condition an activation of prefrontal areas is predominant (Konishi et al. 1998; Smith et al. 2004) and second, the small amount of errors committed in intradimensional shifts did not allow to carry out a comparison between last false and first correct answers.

The cards were presented to the participants as described in Chapter 3. Triggers were set for the number of trials after a shift, the presentation of the cards with additional coding of the shift (intradimensional/extradimensional) that happened before the first trial, and response accuracy (see Figure 4.2 for timeline and trigger coding). Cards were squared with width and height of 2.52'' (6.4cm) and subtended a visual angle of approximately 3.67°. They were presented above the center of the screen for the reference card (the one to sort) and below for the target cards (the ones to choose the sorting rule
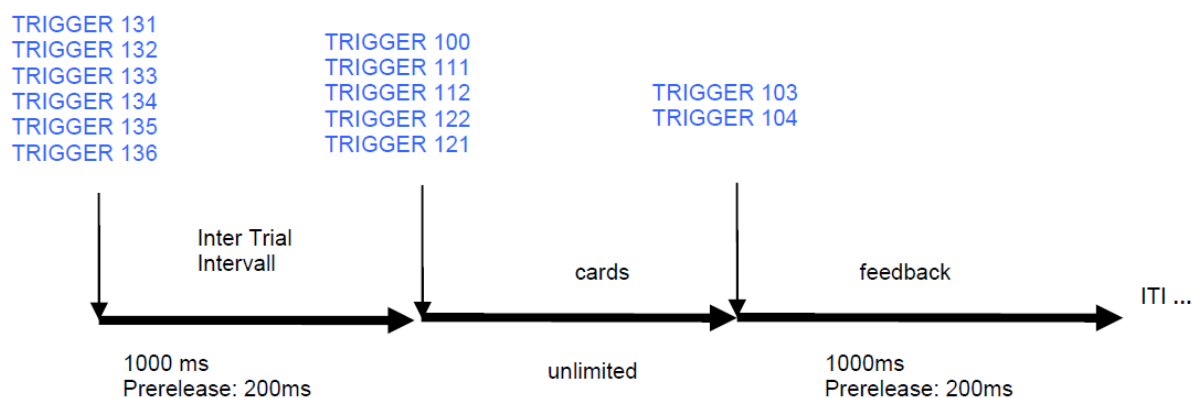
Figure 4.2: time line and triggers set for EEG recordings of the WCST - trigger 13X: trial number, trigger 1XX: shift type - intradimensional shift (shape to shape: 11, color to color: 22), extradimensional shift (shape to color: 12, color to shape: 21), trigger 10X: response (correct: 3, false: 4), trigger 100: beginning of a block → no shift.

4.4 Data Acquisition

EEG was recorded with the Easy-Cap electrode system (Falk Minow Services, Ammersee, Germany) from 27 Ag/AgCl electrodes according to the 10-10 electrode reference system (Chatrian et al. 1988) including the mastoids. All sites were referenced to vertex (Cz). A bipolar electrooculogram (EOG) was recorded horizontally from the epicanthus of both eyes

and vertically from supra- and infraorbital positions of the left eye. Skin and scalp of the participants were prepared with alcohol and gently abraded to achieve an impedance of below 5 kΩ for all electrodes. EEG and EOG were amplified with a 32-channel SynAmps Model 5083 amplifier (input impedance: 10 MΩ; Neuroscan, Inc.) in AC mode. The pass-band was set to 0.05- to 20-Hz (−48 dB/octave roll off); the signals were digitalized at 1000 Hz and stored to hard disk for later analysis.

The EEG was re-referenced to linked mastoids. Eye movement artifacts were corrected semiautomatically based on the algorithm of Gratton et al. (Gratton et al. 1983) and non-physiological artifacts were detected and excluded from analysis by semiautomatic artifact rejection. For both tasks, data were down sampled to 256 Hz and filtered using a 20-Hz (-48dB/octave roll off) digital low pass filter.

4.4.1 Data Processing: ANT

EEG and EOG were epoched off-line into 2400 ms segments starting 200 ms before stimulus onset and ending 2200 ms after stimulus onset. A baseline correction was performed using the first 200 ms as a reference. For each flanker (neutral, congruent, incongruent) and cue condition (no, center, double, spatial) separate averages were computed for each electrode. Difference waves were generated for the three attention networks alertness (no cue – double cue), orienting (centre cue – spatial cue) and executive control/conflict (incongruent flanker – congruent flanker) (see Chapter 3). Grand average ERPs were averaged across all participants as well as across for genetic groups for all cue and flanker conditions as for alertness, orienting and executive control difference ERPs. Visual inspection of the grand average ERPs and point-by-point inspection of effect sizes (Strelzyk et al., in review) of all six group comparisons on all channels and time frames were carried out for the three attention networks. Inspection of EEG data revealed differences between genetic groups in both the alertness and executive control network, but in the orienting network.

Alertness: 900-1000 ms

Difference waves were generated for the whole sample as well as for all four experimental groups by subtraction of ERPs for the double cue condition from the no cue condition. For all group comparisons, T-tests were calculated and effect sizes were estimated subsequently in the Vision Analyzer Software (Brain Products GmbH, Gilching, Germany). Thereafter, data for effect size estimates were exported to the MATLAB software (MathWorks®, Natick, Massachusets) and visualized by a recently developed tool (Strelzyk et al., in review). Based on the visual inspection of effects sizes, we chose to further analyze the timeframe between 900 and 1000 ms. In this timeframe, three of six possible group comparisons revealed considerably high estimated effect sizes at frontopolar to centroparietal positions. Both

conditions of the alertness network were then included separately in the analysis to assess how the genotype groups differ in brain activity between the "alert state" condition (double cue) and the not alerting condition (no cue).

Figure 4.3 shows the ERPS for double cue and no cue trials averaged over the whole sample. Presentation of the double cue elicited an early frontopolar (and frontal) positivity with subsequent negativity that did not show up under the no cue condition. At the same timepoint (about 150 ms after cue presentation), a negativity was detected on parietal and occipital positions. In both conditions, a further positivity arose on frontopolar and less marked at frontal electrodes in both conditions, about 700 ms after presentation of the cue. The second positivity still was more pronounced for the double cue condition than for the no cue condition. After the second positive peak around 700 ms, a negativity showed up with maximal negativity around 1000 ms, followed by a slow positive slope during the ensuing 500 ms. The second positive peak at frontopolar electrodes as well as the following activation pattern was reflected by a negative peak and subsequent positivity at centroparietal to occipital positions.
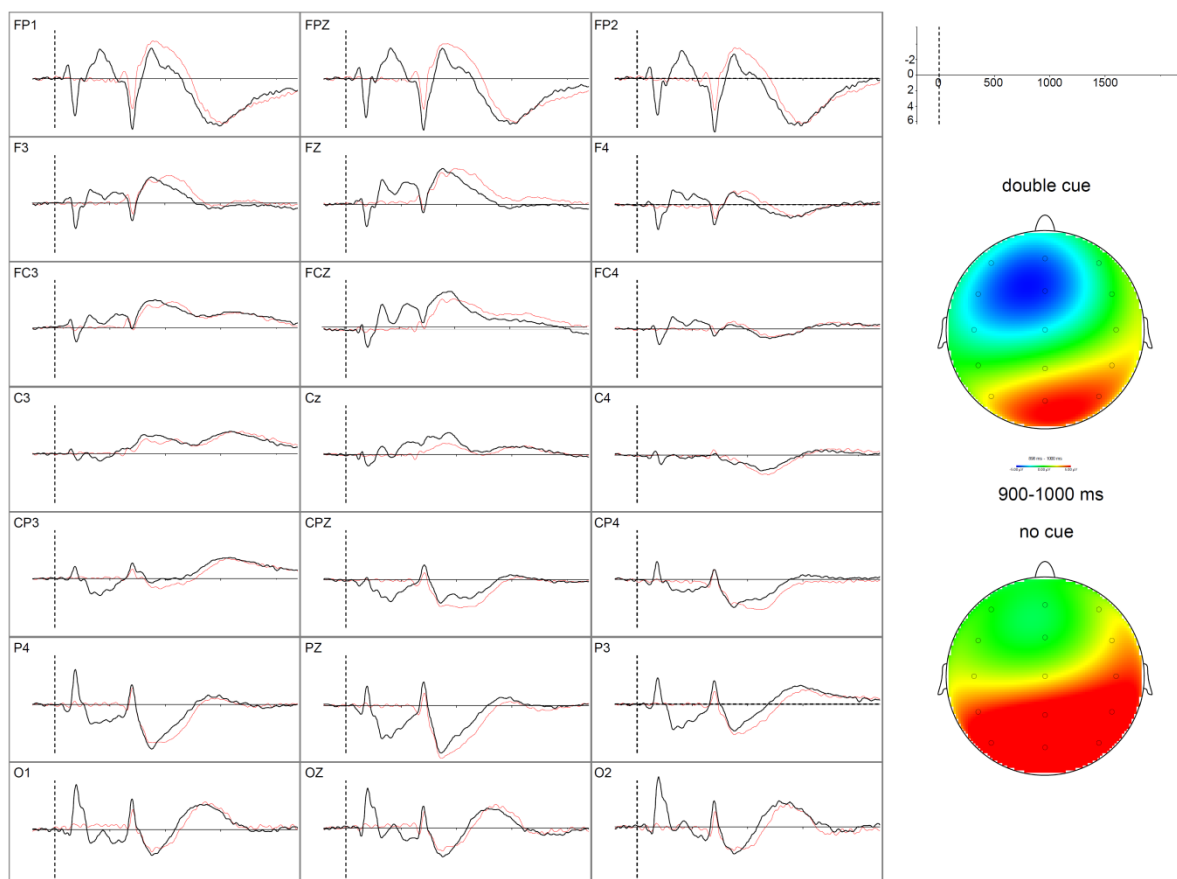
Figure 4.3: ERPs for no cue (black line) and double cue (red line) conditions in ANT, averaged over all genetic groups.

Conflict: 600-800 ms

Data for the executive control network was treated as described for the alertness network. The difference waves were calculated by subtracting the congruent flanker ERPs from the incongruent flanker ERPs. Surprisingly, there were no (significant) differences between the two conditions, as can be seen in Figure 4.4 that shows ERPs for incongruent and congruent flankers, starting with the presentation of the flankers. Like in the double cue condition assessing one part of the alertness network, there were two positive peaks around 150 ms and 700 ms after the presentation of the flankers that appeared in the form of negative deflections at centroparietal to occipital electrodes. Furthermore the negativity and positive slope following the second positive peak in both conditions constitutive for the alertness network were found for the ERPs for congruent and incongruent flankers. Estimated effect sizes of three group comparisons were considerably high in the timeframe between 600 and 800 ms and consequently this interval was chosen for further data analysis.
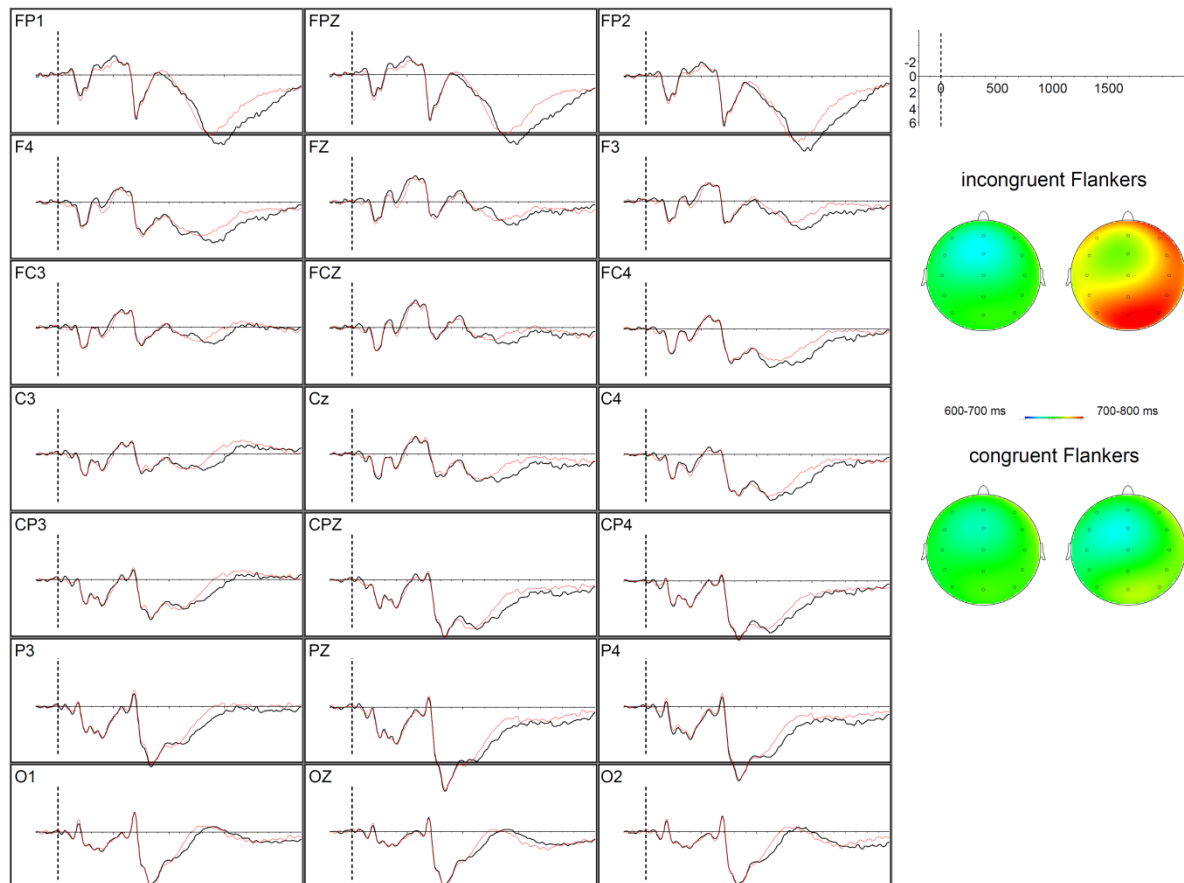
Figure 4.4: ERPs for incongruent (black line) and congruent (red line) flanker conditions in ANT, averaged over all genetic groups.

The aim of this exploratory approach to choose timeframes by visual data inspection was to find effects of the MR genotype on attention measures. Literature of cortisol effects on ERPs is sparse and there is not much known about which ERP components, especially in attention

tasks, may be prominent to be affected by cortisol. In the present investigation, differences between genotype groups, as assessed by estimated effect sizes, were detected in a time window characterized by relative negativity in both conditions assessing the alertness network. For the conditions constitutive for the executive control network (incongruent and congruent flankers), a frontopolar positive peak was found within the chosen timeframe. Based on the cortical spreading of notable effect sizes, for both networks electrodes on and surrounding the midline were chosen and positions from frontopolar to centroparietal were integrated in the analysis.
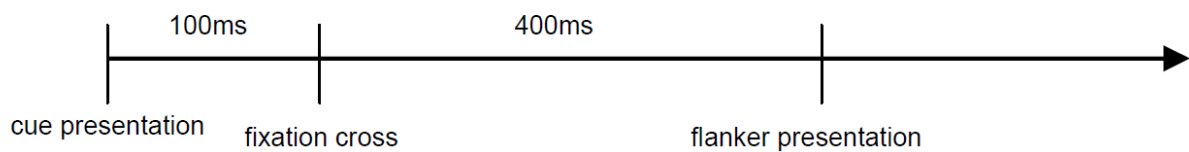
Figure 4.5: time line for the presentation of ANT cues and flankers.

The time windows that were chosen for further analyses were around the time, when subjects react by button pressing with the fingers of the right hand in both networks. The reaction times for each condition of the ANT are given in Chapter 3, relative to the presentation of the flanker row. Reaction times for the double cue and the no cue condition were 616.6 ms (SD= 63.3) and 636.61 (SD= 63.36) respectively. Subtraction of the 500 ms delay after the presentation of the cues from the time window from 900 to 1000 ms results in a timeframe that preceded the subjects' reactions in the alertness network measures (see Figure 4.5). In the executive control network, the ERP component that was chosen started with the reaction to the flankers (RT congruent flankers 597.65 (SD= 62), incongruent flankers 643.7 (SD= 70.22)).

Inspection of the descriptive means, averaged over the cue or flanker conditions revealed an enhanced relative negativity on frontocentral electrodes (middle and left) in both networks, underlining the presumption that genotype differences are related to the motor response of the subjects to the flankers. The most important difference between the networks on a descriptive level was the cortical activity on frontopolar electrodes, which for alertness was characterized by a marked negativity and in the executive control network by a distinct positivity.

4.4.2 Data Processing: WCST

EEG and EOG were epoched off-line into 2195 ms segments starting 200 ms before the response recording and ending 1995 ms after onset of the response. A baseline correction was performed using the first 200 ms as a reference. To detect the moment of rule switching for the WCST, ERPs were time locked to the last false and the first correct reaction in one rule block after an intradimensional or extradimensional shift had taken place. The rationale for this method is that last false trials in this case are 'efficient errors' (Watson et al. 2006) and contribute to the finding of the new rule. First correct responses indicate that the preceding negative feedback has been successfully used to acquire the new sorting rule. Extradimensional shifts were characterized by the change from one category to the other (e.g. shape → color), whereas intradimensional shifts were delineated by a change from one exemplar of a category to another, but of the same category (e.g. red → green, cross → triangle). In 94% of all extradimensional shifts, subjects failed to sort the first card correctly, but then, with the second trial began to sort in accordance to the new rule. As mentioned in the previous chapter (Chapter 3), intradimensional shifts are easier to detect and in 91% of first intradimensional shift trials, cards were sorted correctly. In sum, there were not enough segments for intradimensional last faults to analyze this condition in the same way as extradimensional shifts. Therefore, analysis of ERPs in the WCST was restricted to extradimensional shifts.

The ERP data was treated as described for the ANT alertness and conflict network except that both conditions – last false and first correct trials – were exported directly without calculating difference waves. While for the ANT it was assumed that each of the three attention networks was composed of two conditions, the extradimensional last false and first correct trials are distinct processes of set shifting. Like for the ANT the grand averages for each genotype group were generated and T-tests were carried out for each group comparison for last faults and first correct answers. Subsequently, effect sizes were estimated based on the F values of the T-tests and were inspected visually and by point-to-point inspection. Effect sizes were exported to the MATLAB software (MathWorks[®], Natick, Massachusets) and visualized for more detailed inspection. In both conditions, effect sizes of three group comparisons were sufficiently high for further analysis in the timeframe from 600 to 1000 ms. As can be seen in Figure 4.6, there are opposing amplitudes around 500 ms (first correct answer) and 600 ms (last false trial) for the two conditions. Afterwards, the ERPs take opponent courses and so differences between the conditions could be obscured when the mean activity for the whole timeframe would have been exported for further analysis. That for, the chosen time window was split into two timeframes, the first ranging from 600 to 800 ms and the second from 800 to 1000 ms.
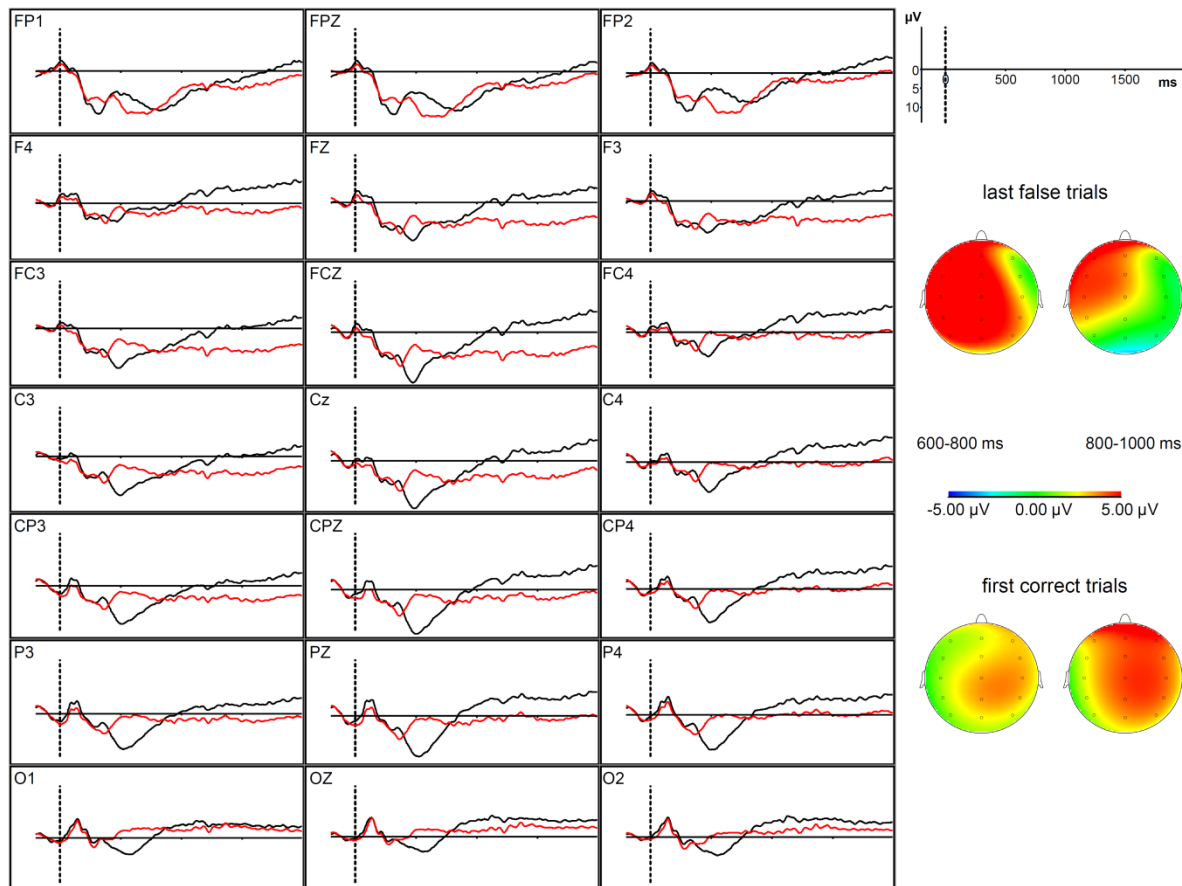
Figure 4.6: ERPs for last false (black line) and first correct (red line) flanker conditions in ANT, averaged over all genetic groups. Amplitudes on frontopolar electrodes under the last false (red line) extradimensional shift trials are opposing the amplitudes under first correct (black line) extradimensional shift trials.

4.5 Statistical Analysis EEG data

ERPs of all conditions of the two tests were analyzed in an exploratory way to find components influenced by the MR genotype (as described above) focusing on mid-frontal areas. ERPs were analyzed with Analyses of Variances (ANOVA) for repeated measures including the dependent variables "condition", "position" (anterior-posterior distribution of electrodes) and "laterality" (right, central, or left electrode) for both tests as well as "time" for the WCST. The between-subject factor "genotype" refers to the four groups constructed, based on the biallelic variants of MR.

Whenever the assumption of sphericity was violated, Huynh-Feldt (HF) corrections (Huynh and Feldt 1976) were applied and only corrected results are reported. For post hoc analyses of significant interactions, the *Dunn's Procedure of Multiple Comparisons* was applied. This test takes into account the amount of comparisons made in the respective interaction but therefore is sensitive to unequal group sizes. One subject (from MR-2GG) had to be taken out of the analysis because of resting artifacts. Further subjects were left out of the analyses that were chosen by their age and/or sex, leading to four equal sized genotype groups of 16

subjects each (MR180V: mean age: 25, *SD*= 3.08, 8 men, 8 women, MR-2GG: mean age: 23.38, *SD*= 2.9, 8 men, 8 women, MR-2GC: mean age: 26.19, *SD*= 4.55, 8 men, 8 women, MR-2CC: mean age: 26.06, *SD*= 2.72, 10 men, 6 women).

4.6 Results ERP Data

4.6.1 ANT - Alertness 900-1000 ms

The alertness network is assessed by the difference between the "no cue" and the "double cue" condition and after visual data inspection possible differences for genetic groups were detected between 900 and 1000 ms after the presentation of the cues.

In the alertness network there is a significant interaction of position, laterality, condition, and genotype ($F_{24/544}$ =1.858, p=.015 HF, η_p^2=.083).

There were two possibilities to disentangle the interaction by the *Dunn's Procedure of Multiple Comparisons*. The first approach comprises the comparison of critical differences between two genotype groups at all electrodes included and for all six possible group comparisons. The second approach is the analysis of the critical difference between the two cue conditions in each of the four genotype groups. Post hoc application of the Dunn's procedure revealed that all of the significant group differences show up at frontopolar to frontocentral electrodes for the presentation of a double cue. In the no cue condition, significant differences in cortical activity between genetic groups are distributed over all electrodes but were most prominent at frontocentral and centroparietal sites (five or six significant group comparisons at FCZ, FC4, CPZ, and CP4). Post hoc analysis of the difference between conditions within the different genotype groups showed that the -2GG, and -2GC group displayed significant differences (at a 1% level) between the no cue and the double cue condition at all electrode positions from frontal to parietal sites (twelve out of fifteen). Amplitude differences between conditions in the 180V group are significant at only ten out of fifteen electrode positions and the MR-2CC group showed differences between the two cue conditions at only five out of fifteen electrodes.

Figures 4.7 a) - d) show the amplitude differences between cue conditions averaged over the hemispheres in the four MR genotype groups. The -2GC group and -2GG participants can be characterized by marked differences between the two conditions with negativity from frontopolar to frontocentral electrodes in the alerting (double cue) condition and rising positivity from frontopolar to centroparietal electrodes in both conditions. The 180V group and -2CC group showed fewer differences between conditions, characterized by less negativity in the double cue condition and a slower rise in positivity from the forehead to centroparietal sites in the no cue condition.
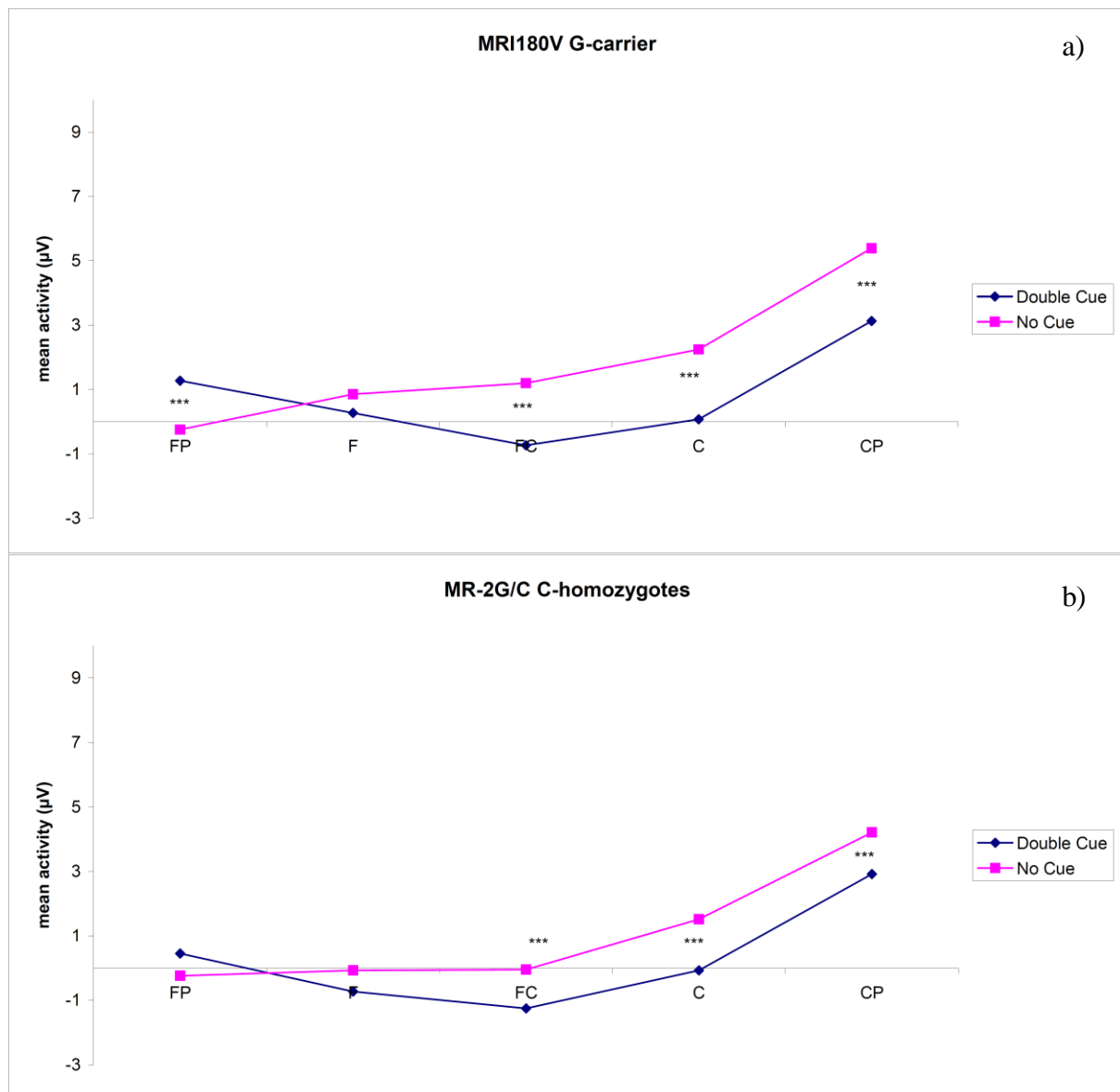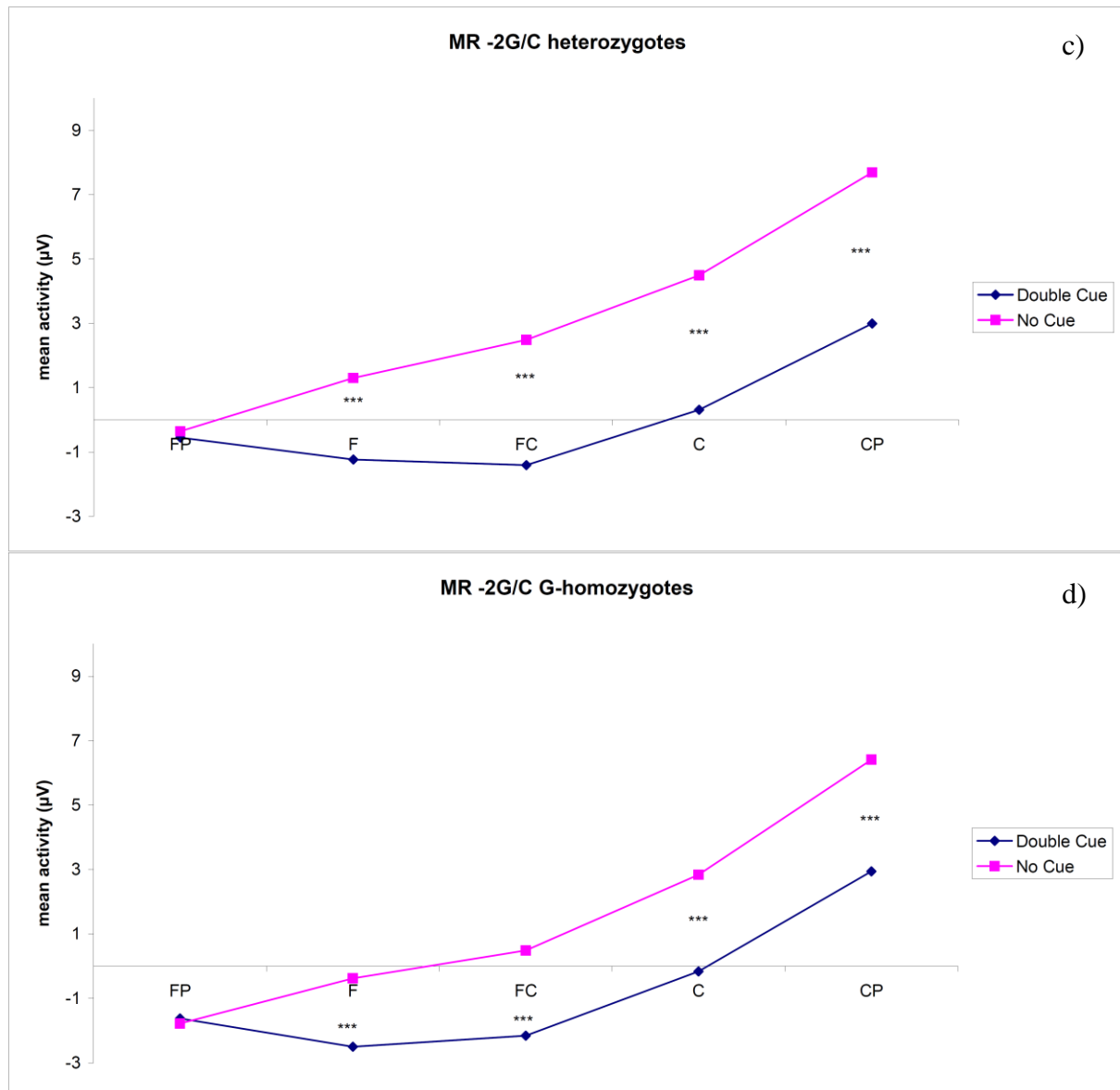
Figure 4.7: Mean amplitude, averaged over hemispheres (µV) for double cue (dark blue) and no cue (pink) conditions between 900 and 1000 ms after cue presentation. (***: d ifference significant on 1% level after application of the Dunn's Procedure of Multiple Comparisons.)

For the chosen timeframe in the alertness network, all main effects were statistically significant ($F_{4/240}$=39.2, p<.0001, η_p^2=.40), laterality ($F_{2/120}$=75.79, p<.0001, η_p^2=.56), and condition ($F_{1/60}$=13.79, p<.0001; η_p^2=.19). Thus, the relative negativity significantly diminishes from frontopolar to frontocentral electrode positions turning into a relative positivity at centroparietal to parietal electrode positions. This posterior positivity was greatest over the right hemisphere and greater in the no-cue compared to the double cue condition.

4.6.2 ANT - Conflict 600-800 ms

In the executive control network, group differences were found in the time frame of 600 and 800 ms. There were significant main effects of electrode position ($F_{4/240}$=17.434, p<.0001,

η_p^2=.23), laterality ($F_{2/120}$=46.369, p<.0001, η_p^2=.44), as well as a significant interaction between position and laterality ($F_{8/480}$=12.196, p<.0001, η_p^2=.17).

We found no significant main effect of condition, indicating no significant amplitude differences between congruent and incongruent flankers, averaged over the whole sample. A visual comparison of the whole ERP responses in both conditions revealed very similar cortical responses across all spatiotemporal areas (see Figure 4.4).

In this network, there was a trend for a main effect of genotype ($F_{3/60}$=2.64, p=.058, η_p^2=.12). Relative positivity was greatest for -2GC subjects independent of the experimental condition and recording site, followed by the 180V group and then the -2CC participants. In the -2GG group, a slight negativity was shown for the executive control network.

Furthermore, there was a marginally significant interaction between condition and genotype ($F_{3/60}$=2.46, p=.071; η_p^2=.11). As for the considerable effect size, a further investigation of MR effects in this network seemed reasonable. Figure 4.8 shows the mean amplitude for incongruent and congruent flankers, averaged over all electrodes. It is obvious why the main effect of condition was not significant. While 180V carrier and the -2CC group showed a slightly enhanced positivity in the congruent flanker condition compared to the incongruent flanker condition, the -2GC group exhibited the opposite direction of activation (incongruent < congruent). The -2GG group, which was the only group that showed slight negativity in the conflict network, did not show a difference in cortical activity between the two flanker conditions.
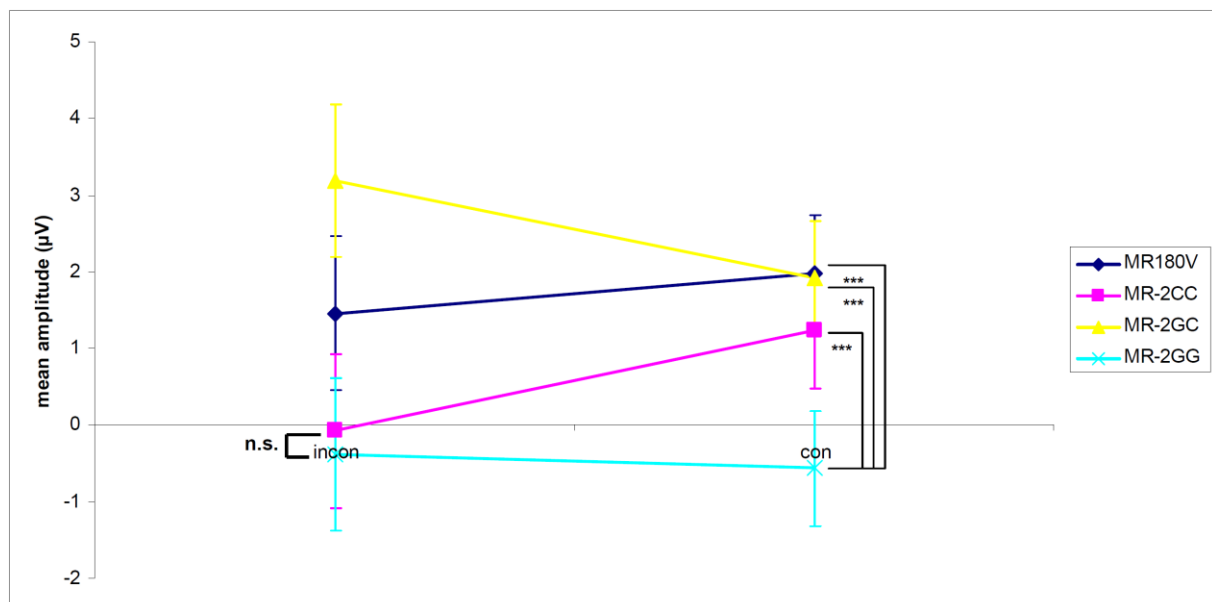
Figure 4.8: Mean amplitude (µV) for genetic groups in both conditions of the executive control network (incon = incongruent flanker, con = congruent flanker). (***: difference significant on 1% level after application of the Dunn's Procedure of Multiple Comparisons.)Error bars indicate the standard error.

Post hoc testing for differences between genotype groups within the flanker conditions showed that in the incongruent flanker condition, all group differences were statistically significant, apart from the comparison of the MR-2GG and -2CC group. In the congruent flanker condition, only three (MR180V minus -2GG; -2CC minus -2GG; -2GC minus -2GG) of six group comparisons showed absolute differences that were significant on a 1% level. Dunn's post hoc test for the analysis of differences between flanker conditions within each of the four genotype groups revealed that amplitude differences, averaged over all electrodes were statistically significant only for the MR-2GC and the -2CC group.

4.6.3 WCST - extradimensional shifts 600-1000 ms

Although there was no significant main effect of condition, a significant interaction of condition and time ($F_{1/60}$ = 9.187, p=.004, η_p^2=.13) showed that conditions have different activation patterns and could be considered as independent when the change over time was taken into account. Post hoc testing of the marginally significant interaction between position and genotype ($F_{18/360}$ = 2.167; p=.10, η_p^2=.10) with the Dunn's procedure revealed significant differences between MR-2GC and both -2 homozygous groups, as well as between MR180V and -2GG at frontopolar electrodes, as shown in Figure 4.9.
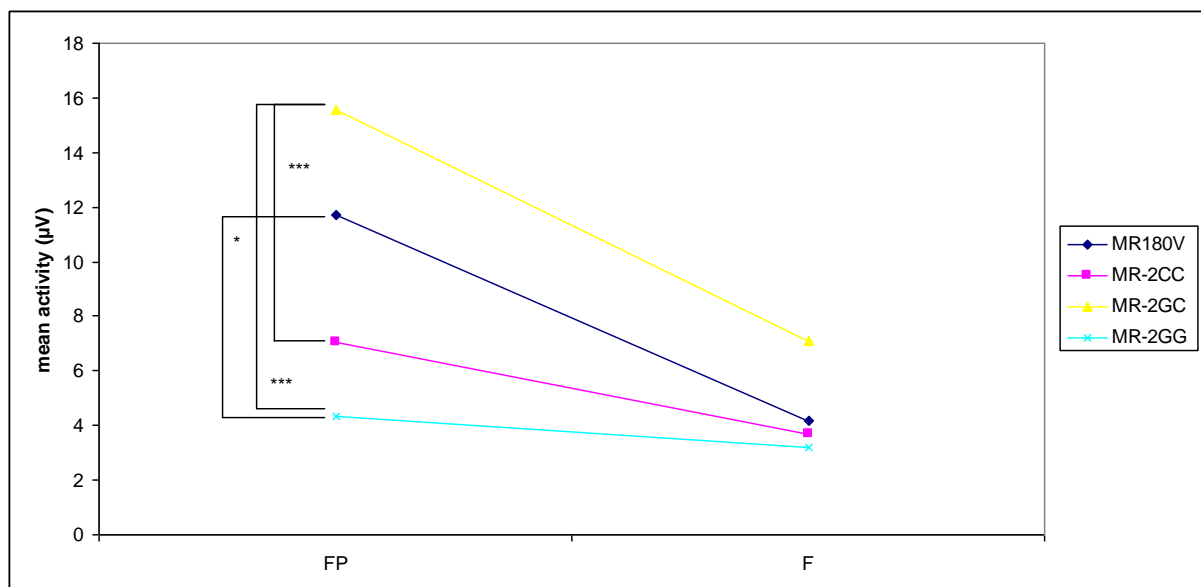
*Figure 4.9: Mean activity (µV) on frontopolar and frontal electrodes, averaged over both conditions and timeframes for the WCST analysis. Note that the mean activity is not referred to as "amplitude" because the whole time window at least in first correct reactions a distinct ERP component could not be described. (***: difference significant on 1% level after application of the Dunn's Procedure of Multiple Comparisons.)*

In addition, the three-way interaction between time, condition, and genotype was marginally significant ($F_{18/360}$ = 2.481, p=.075, η_p^2=.11). Again, as for the moderate effect size, further analyses seemed justifiable. Significant group differences were found in both, last false and first correct responses, and additionally in both timeframes. In each of the time x condition

combinations, the MR-2GC group showed significantly higher positivity averaged over frontopolar and frontal electrodes than the MR-2GG group. Compared with MR-2CC, the MR-2 heterozygous subjects were characterized by higher positivity apart from last false responses from 800 to 1000 ms. Figure 4.10 points out that in general, -2GC subjects showed the highest positivity, followed by MR180V. In both groups, as well as in MR-2CC, positivity declined over the time in first correct trials whereas the -2GG group showed the lowest positivity and did not vary in cortical activity in neither of the conditions.
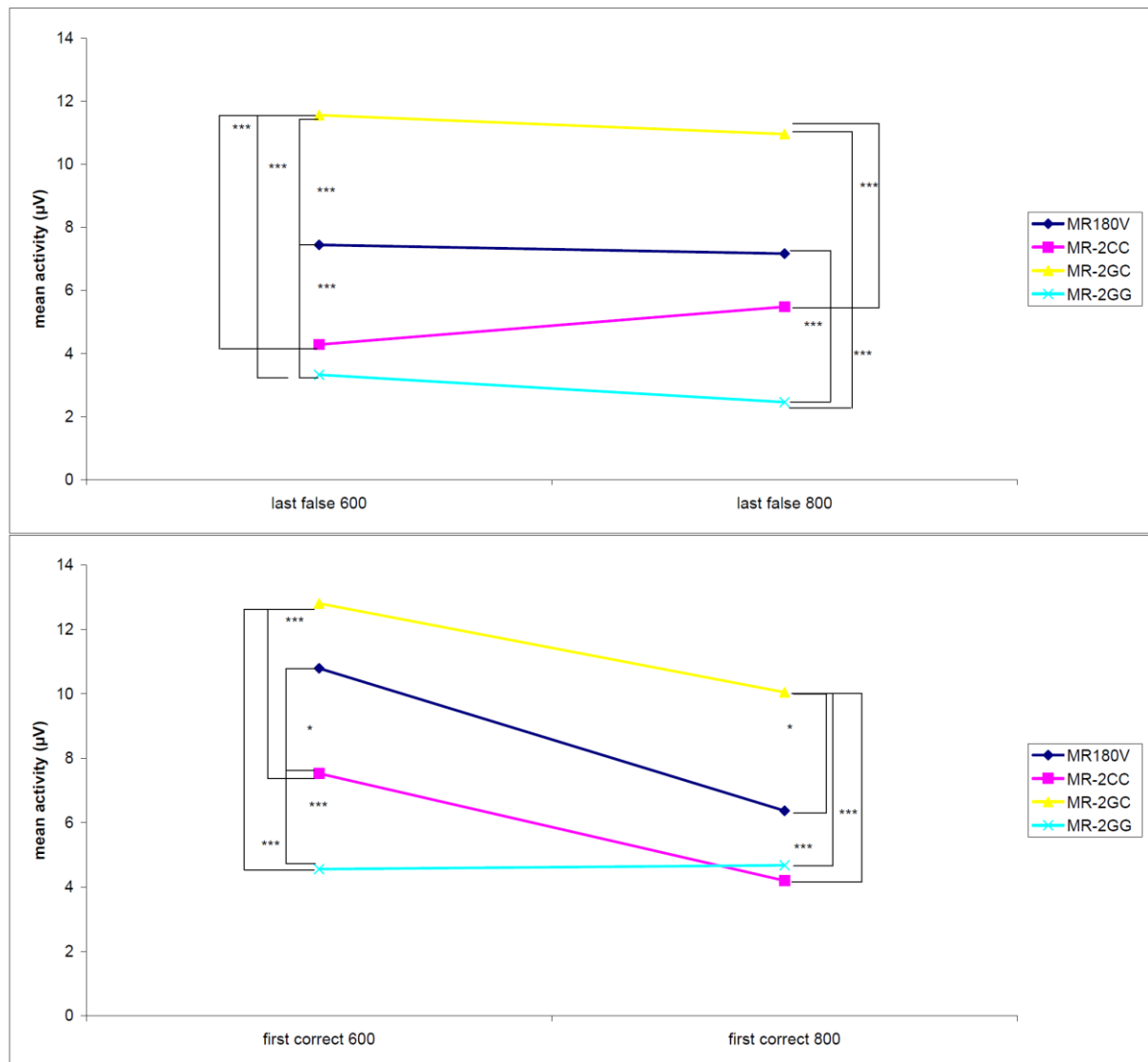
Figure 4.10: Mean activity (µV) of the four genotype groups in last false and first correct trials, averaged over all (six) electrodes, "600" refers to the time interval between 600 and 800 ms, "800" refers to the time interval between 800 and 1000 ms. (***: difference significant on 1% level after application of the Dunn's Procedure of Multiple Comparisons.)

4.7 Discussion

The aim of the present study was to detect relations between two particular polymorphisms (MRI180V, MR-2G/C) in the gene coding for the mineralocorticoid receptor (MR) and cognitive functions in humans. Additionally, the intention was to find spatiotemporal areas in event related potentials (ERPs) for the *Attention Network Task* (ANT) and the *Wisconsin Card Sorting Test* (WCST) that were characterized by different cortical activity between groups of participants that differed regarding their genotype for the MR.

The results revealed a contribution of MR polymorphisms in cognitive processes, such as attention and set shifting. Differences in cortical activity between the investigated genetic groups were found in timeframes which are much later than the encoding or interpretation of the presented cues and can therefore rather be ascribed to processes of behavior initiation for motor responses in the ANT or the processing of feedback which is relevant for subsequent reactions for the WCST. This is of particular interest because it would bear up the hypothesis that the central MR is "essential […] for the selection of a behavioral response" as formulated by de Kloet et al. (1999).

For a normal test performance in WCST, feedback is crucial for response selection in the following trial and can be characterized as a sufficiently complex stimulus. Averaged ERP waveforms for last false trials after an extradimensional shift in our sample depicted a late positive component with centroparietal to parietal maximum at about 450 ms after onset of feedback presentation. A lot of electrophysiological literature has concentrated on a prominent ERP component, namely the positivity occurring around 300 ms (P3 or P300) after the appearance of a rare, unexpected, and task-relevant stimulus. It is modulated by perceptual demands and has been shown that its latency can last up to 1000 ms. Neural generators include HC, amygdala, ventrolateral PFC, and thalamus and are thought to build a cortical and subcortical network that generates this ERP component (Duncan et al. 2009). The P300 is influenced by supraphysiological levels of cortisol in an approach-avoidance task (van Peer et al. 2007) and, moreover, cortisol altered positivity after false trials in an episodic memory task referred to as error positivity (Hsu et al. 2003). Nevertheless, the findings of the present study revealed differences between MR genotype groups on frontopolar electrodes instead of modifying the P300 at centroparietal or parietal sites. Furthermore, the differences in cortical activity between the four genotype groups occurred at a later time window than at the 'normal' peak of the P3 amplitude at 300 ms and lasted for 400 ms.

The topography of activity between 600 and 800 as well as 800 and 1000 ms after an extradimensional shift in the WCST was characterized by marked positivities at frontopolar sites. They were detected for both, last false and first correct trials, but while after last false trials positivity remained relatively stable, after first correct answers frontopolar positivity

decreased distinctly. It has been assumed that in EEG measures, negativity indicates an activation of the given brain area and that positivity, in turn, means deactivation of a particular cell assembly (Westbrook 2000). Frontopolar positivities could thus indicate either relative deactivation of the frontopolar region or – as its pyramidal neurons point to the opposite direction than those of the frontal pole – relative orbitofrontal activation (Westbrook 2000). Frontopolar activation has been associated to rule mapping, as a sub-process of task switching (Rushworth et al. 2005; Travers and West 2008) and, as recently reviewed, it has been shown to be affected in schizophrenia, related to impaired episodic memory performance (Ragland et al. 2009). Altogether, functions of frontopolar brain areas have been shown to react to a wide range of tasks. It has been proposed that the anterior rostral part of medial PFC (mPFC) is involved in processes of self knowledge, person knowledge and "mentalizing" or meta-cognitions, so that this area has been considered as essential for social cognition (Amodio and Frith 2006; Passingham et al. 2010). Furthermore, activity of anterior dorsal mPFC is associated with negative emotion regulation and daytime cortisol measures (Urry et al. 2006) as well as cortisol secretion after a psychosocial stressor (Kern et al. 2008).

The orbitofrontal PFC can be considered as a key component of the reward system and is involved in anticipation of behavior outcome. It is involved in the encoding of the reward value of stimuli in practically all sensory modalities, including social reward (Amodio and Frith 2006; O'Doherty 2007). A very interesting finding in this concern is that orbitofrontal activity is implicated in the regulation of cortisol in response to a psychosocial stressor (Kern et al. 2008; Prüssner et al. 2008) particularly in 'responders' (see below) at the time point of negative feedback (Dedovic et al. 2009). In these experiments the participants have to resolve mental arithmetic tasks that are constructed in a way that they provoke performance failure which is communicated to a larger audience. About 50% of all participants react by enhanced cortisol secretion to this kind of social evaluative threat, referred to as 'responders' (Dedovic et al. 2005). Findings of the research group that implemented this *Montreal Imaging Stress Test* (MIST) consistently showed reduced activity of medial orbitofrontal cortex, the anterior cingulate cortex, and dorsolateral PFC as well as deactivation of HC, amygdala, and hypothalamus linked to psychosocial stress (Prüssner et al. 2004; Wang et al. 2007; Prüssner et al. 2008; Soliman et al. 2008). To date, there is only one recent study using an event-related version of the MIST to disentangle the influence of task components (difficulty of math tasks) and social evaluative components (positive/negative feedback). The authors report of a lower responder rate than for the block design of the task and further detected a deactivation of the frontal pole during the stressful compared to the control condition in all participants, regardless if feedback was negative or not (Dedovic et al. 2009).

It still remains difficult to interpret our results in the light of the different ways of assessing MR function. It would be reasonable to argue that the orbitofrontal cortex is activated during feedback from a cognitive point of view. Nonetheless, a deactivation of the frontal pole might be implicated in the processing of feedback information, maybe referring to the social component of test performance.

Rodent as well as human studies have shown that MR might be involved in actions of the reward systems (Berger et al. 2006; Bogdan et al. 2010). Additionally, in primates MR decreases in expression in prefrontal structures following social stress (Karssen et al. 2007; Patel et al. 2008). The findings of Dedovic et al. (2009) that showed a relative deactivation of the orbitofrontal cortex in responders to the MIST is hardly comparable to the findings of the present study. First, the percentage of responders to the test situation in our study was lower (19% vs. 35%) as in the participants of the investigation of Dedovic et al. (2009). Second, if the orbitofrontal cortex is activated when inhibiting cortisol secretion, it is comprehensible that a stress eliciting task causes deactivation but might be activated during the processing of reward promising situations. The negative feedback in WCST extradimensional trials is informative for the acquisition of a new rule, what might diminish the adversity of negative feedback. Altogether, it can be stated that frontopolar/orbitofrontal activity was generally greater during the last false trials than for first correctly sorted cards. It can be assumed that the information of negative feedback is 'denser' than the information of positive feedback. The previous sorting rule has to be remembered in both conditions but the negative feedback means to switch sets. These differences of information in feedback seem to be monitored better by MR-2CC subjects and the 180V group. In MR-2G/C subjects, the positivity is higher as for the MR-2CC and MR-2GG group and is comparable to the 180V group. An interpretation of this could be that the MR polymorphisms assessed here have differential functions for set shifting either associated with an altered detection of different feedback information for set shifting or connected with the meaningfulness of feedback per se.

For the ANT, group differences appeared at the moment of the motor response to the flanker row in two of the three networks. Only in the orienting network, cortical activity failed to be affected by MR functional polymorphisms. In the alertness network, the differences between genetic groups spread out over multiple electrode positions and were characterized by more or less marked differences between the two alerting cue conditions for the comparison between the no cue and the double cue condition. Notably, the groups which committed significantly more errors in responding to the flankers presented after the cues (see Chapter 3) showed less marked differences of activation between the two conditions. The concerned ERP component is none of the well known and frequently investigated components P1, N1, P2, N2 or P3. In the time window chosen for group comparison, there was a late positivity component which had its maximum over parietal positions. At frontopolar electrodes in the

same timeframe a relatively negative potential was observed. Both had larger amplitudes under the non-alerting no cue condition than in the double cue condition. Additionally, there was a relative negativity in the double cue condition compared to the no cue condition in the time frame from 900 to 1000 ms after cue presentation which compassed frontal to central positions and may indicate an activation of motor cortex in the preparation of a motor response to the (already) presented flankers. The groups differed above all in the difference of activity between the alerting and the non-alerting condition and thus, the fast depolarization of motor cortex areas seemed to be affected by MR genotype.

In the conflict network the group differences did not follow the same pattern. In this comparison of the incongruent and congruent flanker condition, positivity for incongruent flankers was lower for the incongruent condition in the 180V and the –2CC group. In MR-2G/C heterozygotes the positivity was lower in congruent flankers and averaged over both conditions it was higher than in the other groups. The -2GG group showed only minor differences in cortical activation between the two conditions but revealed a marked overall negativity.

In both networks, positivity was lowest on frontal to central electrode positions. Following the suggestion that relative negativity indicates a higher activity of the respective brain areas, we could assume activity differences in primary and supplementary motor cortices shortly before (alertness network), and accordingly during (executive control/conflict network) the reaction. In the alerting network, this effect may be primed by the perception of a rather alerting (double) cue in difference to a less alerting (no) cue.

In the conflict network, we measured frontopolar positivity, which was not detectable for the alerting network. The authors of the ANT suggested beforehand and, subsequently, were able to show activation of the anterior cingulate cortex in fMRI studies during response conflict (Raz 2004; Fan et al. 2005). Activity in the anterior cingulate cortex would heavily produce frontopolar positivity in EEG measures, as it has been identified as generator of the N2. The N2 is an ERP component prominent in error making, characterized by an enhanced negativity over frontocentral sites (van Veen and Carter 2002; Nieuwenhuis et al. 2003; Yeung et al. 2004; Yeung and Nieuwenhuis 2009). There are hints that in normal, healthy subjects, activity in the anterior cingulate cortex are correlated with activity in the orbitofrontal PFC while making errors (Pizzagalli et al. 2003; Holmes and Pizzagalli 2008). But, as we only integrated correct trials in the analysis of ERPs it is not possible that frontopolar positivity was provoked by error processing.

The organization of the PFC and the structural hierarchy of cognitive control still are discussed and remain controversial (e.g. Fuster 1997; Duncan and Owen 2000; Anderson et al. 2004; Bunge 2004; Petrides 2005). One hypothesis is the hierarchical organization from anterior to posterior structures leading from higher order cognitive processes to actions.

Otherwise spoken, the more complex the task gets, the more anterior parts of the PFC will be activated to resolve response conflict. Two experiments have been carried out, which manipulated task complexity and were able to show that in simple reaction competition paradigms, the premotor- and primary motor cortices were activated whereas more frontal areas only became activated with increasing complexity of the task (Koechlin et al. 2003; Badre and D'Esposito 2007). These findings could explain why in the present study associations of MR SNPs with cortical activity were found on distinct cortical regions in the two tests assessing different aspects of executive function. The ANT is a simple stimulus-reaction task (comprising cue conditions that 'prime' reactions on a preconscious level) with only two possible alternatives to answer. In this task, differences between MR genotype groups were found on electrode positions on premotor and primary motor cortices for the double cue condition in the alertness network, shortly before the reaction was made. In the WCST, the group differences were found on frontopolar positions. Averaged over all subjects, the frontopolar electrodes further showed the most distinct ERPs with nearly opposite amplitudes at about 500 ms for the last error and the first correct trial after an extradimensional shift. That might indicate that set shifting requires higher order cognitive control, mediated by either the frontal pole or orbitofrontal cortex.

In the conflict network of the ANT, electrode position did not contribute to the differences in cortical activity between the four genotype groups. However, group differences were more prominent in the incongruent flanker condition, which is considered as the condition that provokes higher response conflict as the neutral or congruent flanker row. Surprisingly, the -2GC group showed less positivity in the congruent than in the incongruent condition. If less positivity/more negativity means higher activation of the measured area (Westbrook 2000), this would suggest that in the -2GC group the congruent flanker row would induce a higher response conflict than the incongruent flankers.

The results of the analysis of EKPs for parameters of executive control and alertness are promising findings for the interpretation of MR function in human cognition. We were able to show that prefrontal cortical activity is associated with two MR polymorphisms during processes that contribute to attention processes and are related to cognitive flexibility. These findings are in line with previous rodent studies and underpin the point of view that cortisol can affect distinct cognitive operations by acting via its two central receptors. Further investigations are needed to shed light on the functionality of MR polymorphisms in human cognitive processes that may be related to the activity or reactivity of the HPA axis and thus may be linked with the vulnerability of developing psychiatric diseases.

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CHAPTER 5

Mildly Stimulated Cortisol Levels in the Afternoon are associated with Mineralocorticoid Receptor Polymorphisms in a Sex Specific Way

Chapter 5: Mildly Stimulated Cortisol Levels in the Afternoon are associated with Mineralcorticoid Receptor Polymorphisms in a Sex Specific Way

Background: The role of the mineralocorticoid receptor (MR) for HPA axis activity and reactivity has extensively been investigated in rodent models, suggesting an influence on basal as well as challenged cortisol levels. In humans, MR polymorphisms have been shown to be associated with parameters of challenged HPA axis activity.

Methods: In the present study, we assessed salivary cortisol measures in a sample of 72 young healthy participants, genotyped for two common polymorphisms in the MR gene (MR-2G/C, MRI180V). Saliva samples were provided for the assessment of daily cortisol secretion on two consecutive workdays as well as during a lab visit for an electroencephalography (EEG) test session.

Results: During the test session, male participants of homozygous for the C allele of MR-2G/C) and MR-2G/C heterozygous men showed raising cortisol levels, while male carriers of the 180V allele were characterized by slightly elevated cortisol levels at the beginning of the session. No associations were detected between the genotype and daily cortisol measures.

Conclusions: Despite the small sample size, the results for an association between the genotype and mildly stimulated HPA axis activity indicate a role of the MR in stress reactivity. These differences seem to be linked to either the anticipation or the interpretation of a situation as potentially stressful.

5.1 Introduction

The physiological stress reaction is controlled by suprahypotahlamic brain regions whose influences on HPA axis regulation have been outlined in the Chapter 2 (2.3: Neurocircuitry of Stress). At the latest since 1985 it has become clear that in those central structures there are two receptors binding to corticosteroids. The MR (NR3C2), which in peripheral tissues rather binds to aldosterone, has a ten fold higher affinity to cortisol in brain then the second receptor – the GR (NR3C1), which in turn is more prevalent in central regions than MR and so has a much higher capacity to bind cortisol as its ligand (Reul and De Kloet 1985). In peripheral tissues MR binds to aldosterone and is thus the main target for mineralocorticoid actions on salt and water retention. This mechanism is mediated on a pre-receptor level by co-localisation of the MR with 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), an enzyme that converts cortisol to cortisone. Cortisone, in turn, is not able to bind to the MR (Viengchareun et al. 2007). MR can bind to progesterone but if progesterone has agonistic or antagonistic action, seems to depend on the cellular context and it is not yet clear to which processes it contributes (Quinkler et al. 2002; Quinkler et al. 2003). Further the MR binds with high affinity to the exogenous corticosteroids dexamethasone, deoxycorticosterone or fludrocortisone (Viengchareun et al. 2007).

Due to their biochemical features it has been thought that MR is responsible for the tonic inhibition on diurnal cortisol secretion whereas GR only binds at peak levels or ongoing stress reactions and exerts a negative feedback response to high circulating corticosteroids as described in detail in the Chapter 2.2.2 (De Kloet and Reul 1987). Meanwhile, there is accumulating evidence that this view has been too simplistic. Pharmacological blockade of the MR in rodents (Ratka et al. 1989; Reul et al. 1997; Gesing et al. 2001; Pace and Spencer 2005) have pointed to an active role of MR in the inhibition of an HPA axis response to a stressor. Results in studies which block the (central) MR differ depending on the antagonist chosen for blockade (Reul et al. 1997) or the type of stressor used (Pace and Spencer 2005). Furthermore, studies working with genetically modified animals partly contradict the finding of a cortisol enhancing effect of MR blockade at the diurnal nadir (Berger et al. 2006) and on stimulated HPA axis reactivity (Lai et al. 2007; Rozeboom et al. 2007). Only female mice overexpressing MR in the forebrain show a moderate but significant suppression of the corticosterone response after restraint stress (Rozeboom et al. 2007). Further, a non-habituation of the corticosterone response to fear conditioning has been shown only in female mice down regulated for MR in the forebrain (Brinks et al. 2009). These results indicate that high MR expression in limbic structures has an attenuating effect on stimulated levels of corticosteroids. The differences between studies using a pharmacological approach and those using genetic modelling of brain specific MR already make obvious that there may be marked differences between the acute administration of a MR antagonist and persistent

changes of the receptor itself. That MR can mediate corticosterone secretion by fast feedback in a pulsatile manner has recently been shown by Atkinson et al. (Atkinson et al. 2008) in rats and that it may rapidly inhibit basal HPA axis activity has been observed in humans (Otte et al. 2003).

In humans, there are some studies assessing the effect of pharmacological blockade of MR on cortisol measures (Dodt et al. 1993; Born et al. 1997; Deuschle et al. 1998; Young et al. 1998; Heuser et al. 2000; Young et al. 2000; Arvat et al. 2001; Young et al. 2003; Wellhoener et al. 2004; Mattsson et al. 2009) showing an overall enhancement of cortisol secretion after antagonist administration (basal and stimulated) as well as discriminative effects for depressed patients (Young et al. 2000; Young et al. 2003) or obese men (Mattsson et al. 2009) compared to controls.

To date there are only two studies investigating the effect of MR polymorphisms on cortisol levels in humans showing that MR SNPs modulate the repression of the CAR after dexamethasone treatment as well as the HPA axis reactivity to a psychosocial stressor (DeRijk et al. 2006; van Leeuwen et al. 2010). The first studied variant is the polymorphism rs5522, in the following referred to as MRI180V, located in the translated region in exon 2 of *NR3C2* and it leads to an isoleucine to valine change in position 180 of the MR protein. It is characterized by an A to G transition with the G allele being the minor allele. The other polymorphism rs2070951 is located in the untranslated region two base pairs downstream of the first start codon and it is characterized by a G/C transition and will be referred to as MR-2G/C. It has been shown that both polymorphisms affect transactivational capacity of the MR *in vitro* with either cortisol or dexamethasone used as ligand (van Leeuwen et al. 2010).

As pharmacological manipulation of MR leads to changes in basal cortisol levels and as MR I180V and -2G/C have previously been shown to be associated to cortisol responses to challenge we sought to study if these two polymorphisms are associated with cortisol levels during a potentially stressful EEG test session taking place in the afternoon at low levels of cortisol. Additionally, we assessed brief daytime cortisol profiles to control for a contribution of MR on unstimulated cortisol levels in order to accomplish previous observations of our research group on MR function (DeRijk et al. 2006; van Leeuwen et al. 2010).

5.2 Subjects

From the previously genotyped population of young healthy subjects, 72 right-handed participants (38 man, 34 women, mean age: 25.88, *SD*= 4.823) were invited to the psychophysiology lab of the Trier University to take part in the main study. All subjects had normal or corrected to normal vision. The experimental groups are described in detail in Chapter 3 and were built based on the participants' genotype for the MR gene. The MR180V group was heterogeneous for MR-2G/C but contained all carriers of the rare G allele of

MRI180V (10 men, 9 women, mean age: 26.33, SD= 5.65). The MR-2CC group (10 men, 6 women, mean age: 23.6, SD= 2.85) is built of homozygous carriers of the C allele, MR-2GC (9 men, 9 women, mean age: 27.56, SD= 6.25) were all heterozygous for MR-2G/C and G homozygous participants are referred to as MR-2GG (10 women, 9 men, mean age: 25.95, SD= 3.03). These three groups last mentioned were homozygous for the A allele of MRI180V.

5.3 Experimental Procedure

Participants were invited for an EEG session including the accomplishment of two cognitive tasks (see Chapter 3). During the lab visit five saliva samples were obtained. The first one was collected five minutes after the participants sat down for EEG preparation (for details see Chapter 4). The remaining samples were collected every 10 to 15 minutes (sample 2 to 5), adapted to breaks between test blocks and at the end of the session. Due to the different preparation times, accumulated reaction times and pauses of the participants, the time between two samples varied between subjects. The contribution of pauses after test blocks and between the two tests only differed minimally between subjects. Only the time for preparation of the subjects lead to a longer interval between the first and the second sample in the range of about 20 to 40 minutes.

At the end of the test session, subjects were provided with two sets of saliva sampling devices to collect samples on two consecutive work days. The subjects were instructed to collect samples directly after awakening as well as 30, 45 and 60 minutes afterwards to assess the cortisol awakening rise (CAR). Further samples for a short daytime profile were taken at 8:00 and 11:00 a.m., 3:00 and 8:00 p.m.

The protocol was approved by the ethics committee of the University of Trier. All participants gave written informed consent prior to the sampling of mouth epithelial cells for DNA isolation. Participants received financial compensation either only for the DNA sampling or in addition for the following tests.

5.3.1 Saliva sampling and cortisol analysis

Saliva was collected using a special device for salivary cortisol analysis (Cortisol Salivette® Sarstedt, Nümbrecht, Germany) according to the manufacturer's protocol. Participants were told to store samples collected at home at 2 to 8° C until bringing them to the laboratory where they were kept at -20° C. Samples from EEG sessions were stored directly at -20° C until analysis. The biochemical analysis was carried out using a time resolved immunoassay with fluorescence detection (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay - DELFIA) described elsewhere (Dressendorfer et al. 1992). Intra- and interassay variability of the assay was less than 10 and 12%, respectively.

5.4 Statistical Analysis Cortisol

To analyse cortisol data ANOVAs for repeated measures (to assess the effects of time) were used to measure the effect of genotype controlling for sex. The cortisol secretion during test session (EEG), the CAR and the short diurnal profile were assessed in three different models. In order to further analyse a detected time x sex x genotype interaction in the cortisol levels during the EEG session, we implemented the Dunn's post hoc procedure of multiple comparisons. For the analysis of cortisol secretion during EEG session, four subjects had to be omitted because of missing data. Seven participants had to be excluded from the analyses of the CAR and the short daytime profile because of missing data or late wakening (confounding of CAR and short daytime profile).

In order to classify subjects as either cortisol 'responders' or 'non-responders' to the potentially stressful EEG test session, a difference of at least two nmol/l between the lowest level cortisol sample and the individual peak was defined as a cortisol response to the EEG situation. To check if responders were equally distributed over the four genotype groups and the two sexes a χ^2 test was carried out. An additional ANOVA of repeated measures was computed to test if the rise in cortisol was significantly larger than in non-responders with "responder" as between-subject factor and sex as covariate. Apart from the Dunn's procedure, analyses were accomplished using the software SPSS 15.0 (SPSS Inc., Chicago, IL, USA) Dunn's procedure was calculated in MS Excel according to Dunn (Dunn 1961).

5.5 Results

Regarding cortisol levels during the EEG session there were no significant main effects of genotype group or sex neither interactions between genetic group and sex or interactions between genotype or sex and time on salivary free cortisol. A significant effect of time ($F_{4/240}$= 2.923, p= .022, η_p^2= .046) indicates a general rise in cortisol during the lab visit for the whole sample. As the effect size for the interaction between time x sex x genotype ($F_{12/240}$=1.747, p= .058, η_p^2= .08) was higher than the size of the time effect alone, we applied the Dunn's post hoc test of multiple comparisons to identify the genotype groups with marked sex differences. As shown in Figure 5.1 a), cortisol response curves in female and male carriers of the MR I180V G allele are significantly different for the first two timepoints. While male participants with this genotype showed relatively high pretest levels (at timepoint EEG1) and a continuous subsequent decline, female subjects displayed lower initial levels followed by a cortisol increase. In participants homozygous for the C allele of MR-2G/C only men showed a clear rise in salivary cortisol while female subjects carrying this genotype stayed at a relatively low level. In the -2GC group men had stable but slightly higher levels than women (approximately 1 nmol/l). In -2GG subjects there were no significant differences between men and women. Significance on a 1% level for the comparison of men and women is

reached for time point EEG2, 3 and 4 in the -2CC and -2GC group and on time point EEG1 and 2 in the 180V group (see Figure 5.1).
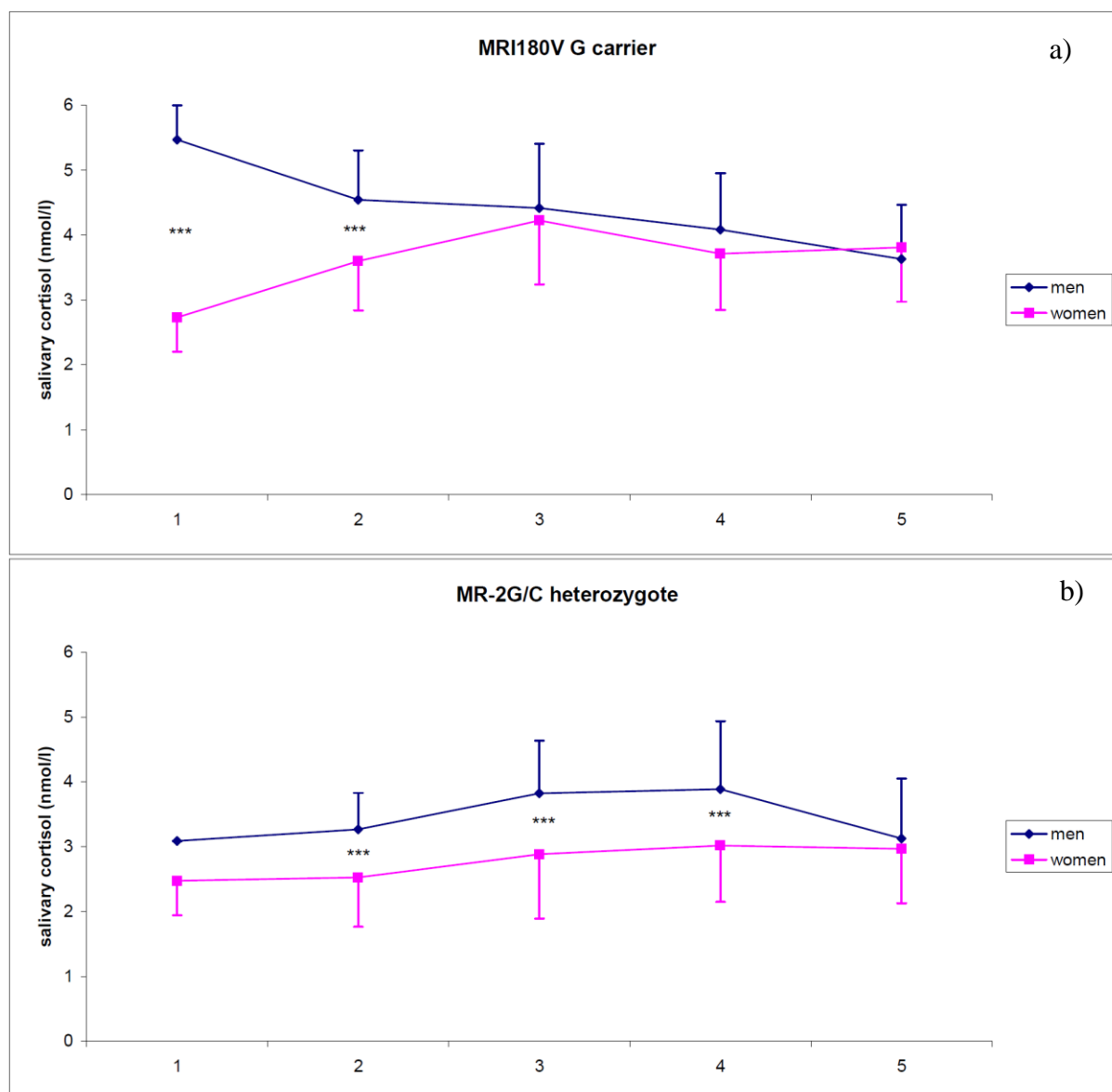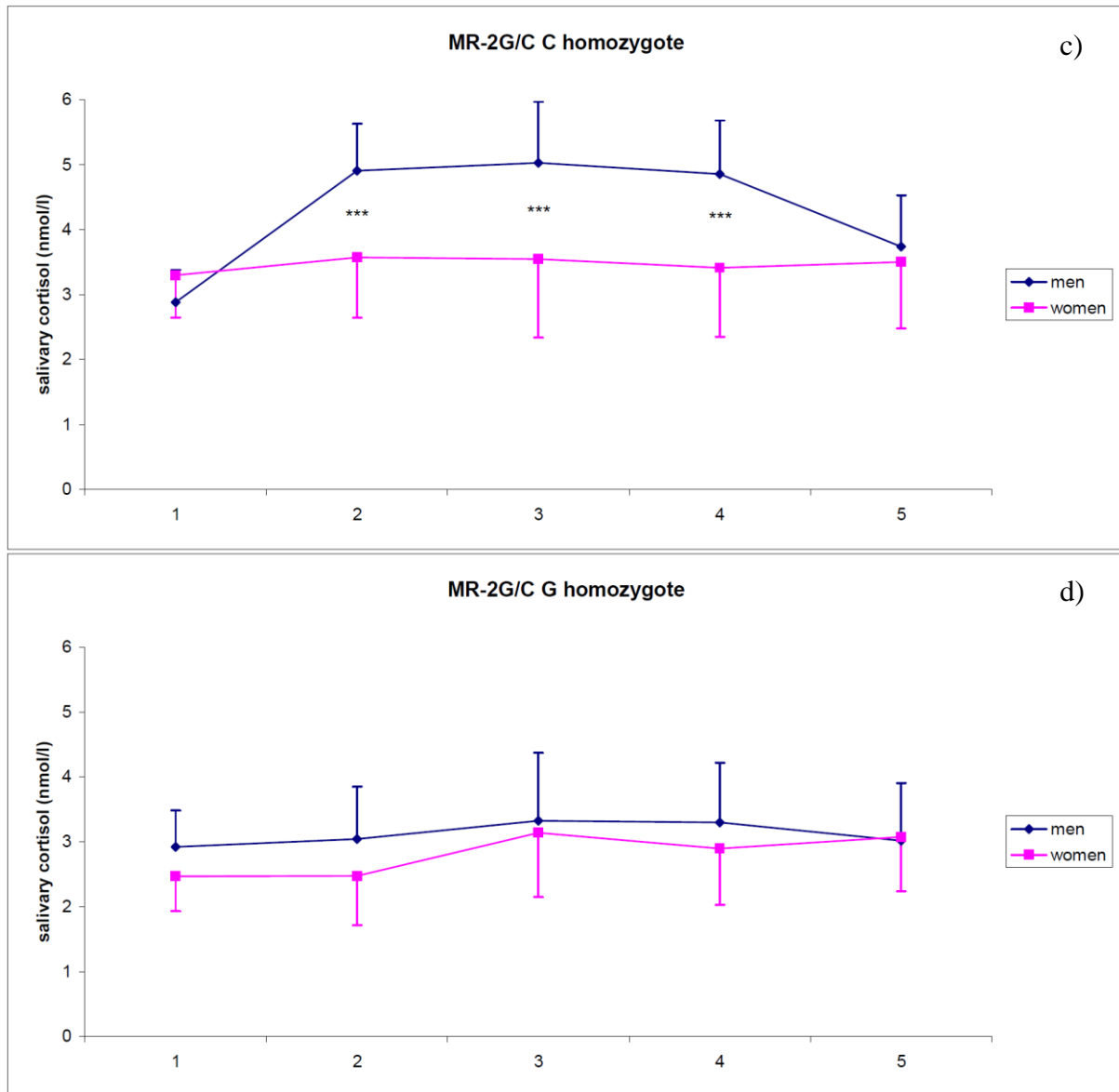
Figure 5.1: Cortisol profiles of sex differences in the four genotype groups during the EEG test session. (*: absolute difference exceeding the critical difference for p=.01 following Dunn's Procedure of Multiple Comparisons). Error bars indicate the standard error.**

We found 19 responders (12.92%) out of 68 participants and they were equally distributed over the four genotype groups (χ_3^2=1.639; p=.651) and across both sexes (χ_1^2=0.014; p=.905). As expected, the classification in responders vs. non-responders resulted in significantly different cortisol response curves between these two groups with a significant main effect responder ($F_{1/64}$=31.904, p<.0001, η_p^2=.38) as well as significant interaction of time x responder ($F_{4/208}$=32,963, p<.0001, η_p^2=.388) and time x responder x sex ($F_{4/208}$=3.426, p= .01, η_p^2= .062). A significant complete interaction of time x sex x genotype x responder ($F_{12/208}$=2.207, p=.013, η_p^2=.113) indicates that the differentiation between responders and non-responders still has an influence on the interaction of time, sex, and genotype described above. But even after having integrated this differentiation in the

analysis, the interaction of time x sex x genotype remains marginally significant and has a comparable effect size ($F_{12/208}$=1.632, p= .085, η_p^2= .086). The Dunn's post hoc procedure was not applied here because it is sensible to different group sizes and by integrating the factor "responder" into the analysis, group sizes differed from 1 to 9 participants per cell.

We did not detect a significant association between MR genotype and the CAR or the short cortisol daytime profile. There were no significant main effects of MR genotype group or interactions with time or sex (all p>.148). Independent of genotype the ANOVA procedure for CAR samples revealed a significant time x sex interaction ($F_{3/171}$=5.702, p=.001, η_p^2=.091) with females showing a comparably high but prolonged morning rise in cortisol (see Figure 5.2). This effect is consistent with previous studies (Prüssner et al. 1997; Steptoe et al. 2000; i.e. Polk et al. 2005; Prüssner et al. 2008).
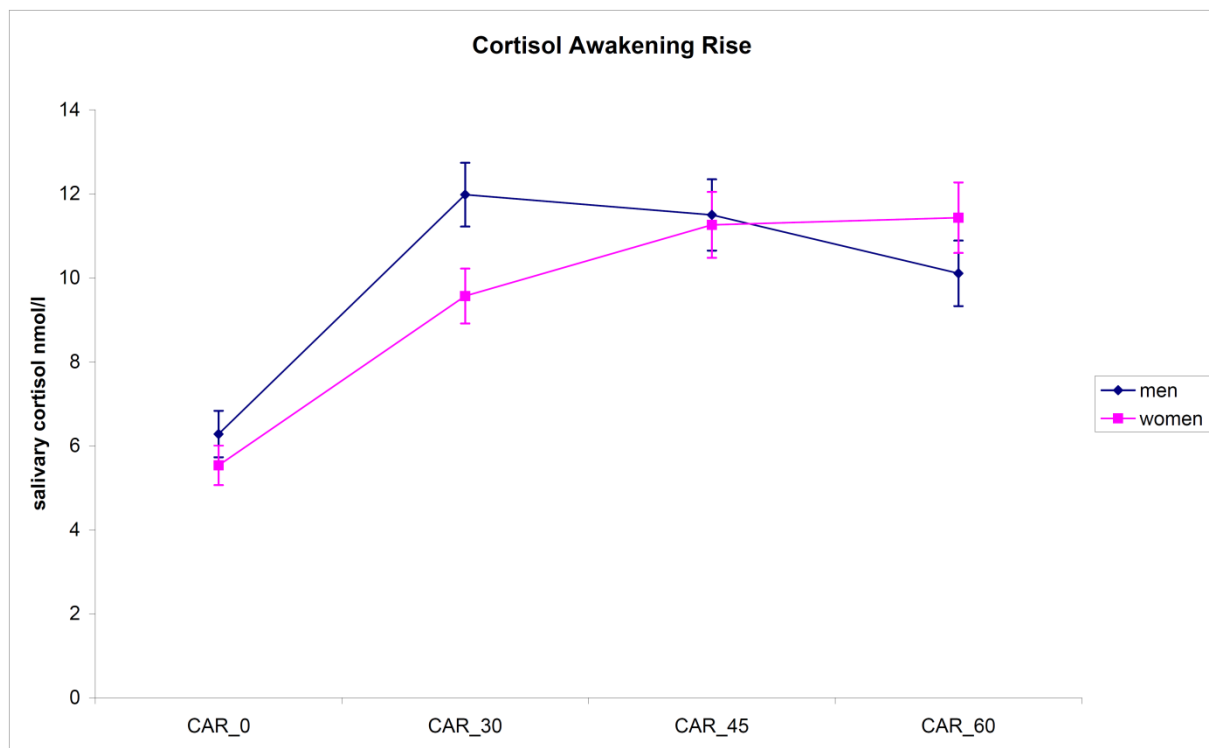
Figure 5.2: Free cortisol in saliva for the cortisol awakening response in male and female participants. Error bars indicate the standard error.

5.6 Discussion

In the present study, the main interest was to characterize two MR SNPs regarding their influence on cognitive functions. More specifically, we hypothesized that the MR would have an impact on cortical EEG activity in tasks that assess executive functions. Because previous studies have shown that the MR has an impact on daytime cortisol levels (Deuschle et al. 1998; Young et al. 1998; Young et al. 2003) and MR polymorphisms have been shown to

influence the acute stress response (DeRijk et al. 2006; van Leeuwen et al. 2010), we integrated measures of salivary cortisol in our study. Sample devices were used on two consecutive workdays and during the EEG session to investigate possible influences of MR polymorphisms on daytime measures and a mildly stimulated HPA axis.

In our sample, we found an interesting interaction between time of sampling, sex of the subjects and the MR genotype on salivary free cortisol secretion during the test session. Although the association was not significant, the effect size was reasonably high to further elucidate the found association using Dunn's post hoc procedure. We were able to show that in three of the four genotype groups, men and women differed significantly in cortisol levels of at least two timepoints. In previous studies, de Rijk and co-workers have shown in male subjects an enhanced salivary cortisol response to a psychosocial stressor of 180V carriers compared to 180I homozygotes (DeRijk et al. 2006). De Rijk et al. were able to show that carriers of the rare (G) allele of MRI180V show enhanced secretion of cortisol after participating in the *Trier Social Stress Test* (TSST), a stress inducing paradigm that provokes levels of cortisol that largely exceed values by which the MR is occupied and are in the range of GR activating levels. As they controlled for GR polymorphisms an influence of this receptor could be excluded. Another study was carried out by van Leeuwen et al. in which the impact of the MR-2G/C and MRI180V polymorphism on the CAR and the dexamethasone induced suppression of the CAR were assessed. They reported differences between genetic groups dependent on the sex of the subjects only for suppressed cortisol levels after awakening, after the system had been challenged by dexamethasone (van Leeuwen et al. 2010). Regarding the dexamethasone suppression effect, van Leeuwen et al. found a pronounced sex effect in -2GG subjects while this is the only genotype were we found no significant difference between sexes at all. In our experiment the differences between -2CC men and women were statistically significant at time points EEG 2,3 and 4, indicating a transient enhancement of cortisol secretion during testing only in men (see Figure 5.1). In the 180V group we see a considerably high difference between men (5.47 nmol/l) and women (2.73 nmol/l) at the first saliva sample.

It is difficult to compare the results of a challenged HPA axis from van Leeuwen et al. with the present study in terms of comparing the genotype directly. The suppression of the CAR after dexamethasone treatment is a GR mediated negative feedback mechanism on HPA activity (Stevens et al. 2004) and includes the involvement of dexamethasone, a potential MR agonist (Rupprecht et al. 1993; Grossmann et al. 2004), whereas the group differences in our sample are detected during a mild stimulation of the system, not reaching GR action relevant cortisol levels. Comparably to the previous investigation of MR function, we didn't find significant associations between the two MR polymorphisms investigated and the CAR. Further, the short daytime profile lacks association with MR polymorphisms. In sum, we

replicated the results of our research group. For both, GR and MR, there is no evidence for HPA axis influencing functions after awakening or during the course of the day (van Leeuwen et al. 2010; Kumsta unpublished data). Cortisol levels in the afternoon and evening show very low or no estimated heritability and morning levels around awakening have been shown to be only moderately influenced by genetic factors (Wüst et al. 2000; Bartels et al. 2003; Kupper et al. 2005). On the other side, the CAR is influenced by different psychosocial factors as investigated in a recent review (Chida and Steptoe 2009) and those may even have effects in a sample of young, healthy adults.

To assess an association of interindividually highly different trait measures like the CAR, the sample investigated here is probably too small. Compared to the previous study investigating effects of MRI180V and MR-2G/C, we investigated a third of the number of participants. The impact of single genes in a widely distributed system of high connectivity normally requires sample sizes even bigger then those investigated by van Leeuwen et al. and effect sizes in genetic association studies are known to be low. The main focus of the investigation presented here was to find possible effects of MR polymorphisms on brain activity during performance of tasks that assess executive functions. Because tested conditions are frequently repeated, even small effects can be found with relatively small group sizes. Therefore, it is encouraging to see that we found effects of MR SNPs on mildly stimulated afternoon levels in our sample. Commenting on the lower levels of salivary cortisol in woman during the EEG session, it has to be mentioned that all women took ethynyl estradiol containing oral contraceptives which are known to enhance the level of corticosteroid-binding globulin (CBG) (Wiegratz et al. 2003). The found results are of rather exploratory character and have to be treated with caution. Nevertheless, they offer the opportunity to develop hypotheses about the association between MR polymorphisms, cognition and the central regulation of HPA axis.

Considering the EEG set up as a potentially moderately stressful situation the response of -2CC men (and to a certain extent responses of 180V women) represent an endocrine stress response to the test situation. In contrast, 180V men showed already relatively high prestress levels and a slow subsequent decline during the session. Given the small effect size it is certainly highly speculative to discuss these differences but looking at the possible mechanisms nevertheless can be interesting. Different stressors have been shown to affect expression of the MR in central tissues (de Kloet et al. 1986). Those changes seem to be region and sex specific (Karandrea, Kittas et al. 2002) and associations with the action of estradiol/estrogen and progesterone have been reported to affect these changes (Carey et al. 1995; Castren et al. 1995). On the other hand MR has been shown to bind progesterone, which can have agonistic or antagonistic function depending on the cellular context (Viengchareun et al. 2007). Furthermore, it has to be considered, that genetic polymorphisms

can affect acute system functions in different ways. The I180V lies in the N-terminal domain of NR3C2 and affects transactivational function of the MR with cortisol but not with aldosterone *in vitro* (DeRijk et al. 2006). The MR-2G/C SNP was tested with cortisol and dexamethasone and could be shown to modulate transactivation as well (van Leeuwen et al. 2010). This could account for downstream changes in transcription targets of the MR and by that leading to long lasting changes in signal transduction over the time leading to a different pattern of sex effects for the MR variants.

Moreover, MR has rapid non-genomic actions on corticosterone secretion (Atkinson et al. 2008). In our experiment the cortisol measures were frequent and showed the variation in unbound salivary cortisol levels for about an hour and a half. It is therefore possible that we assessed in part rapid non-genomic functions of MR. These are mediated via a membrane receptor able to bind to corticosteroids and which is thought to be expressed from *NR3C2* (and/or *NR3C1*) and underlies transcriptional or post-translational differentiation (Stahn and Buttgereit 2008; Di et al. 2009). To date we can not exclude that the MR polymorphisms assessed in this study affect rapid MR signalling and that they account for sex specific group differences between genetic groups. It should be noted that MR, like GR, is regulated and activated in a tissue specific manner, depending on the cellular context (Yang and Young 2009). Consequently, polymorphisms in the gene may also have tissue specific effects. For example male MR 180V carrier came in with already elevated levels of circulating cortisol, which could be interpreted as a prefrontal driven influence of anticipation of the EEG session. The -2CC men, on the other hand, showed a relatively brief stress response to the test situation, which could be due to interpretation of situational cues. In both it is likely that the prefrontal cortex and the limbic system are involved in the individual perception and evaluation of the EEG session – brain regions that have been shown to express MR (Watzka et al. 2000; Xing et al. 2004) and that build a network reacting to the perception and evaluation of novelty.

In our sample we find both SNPs in HWE what is inconsistent with the results of a previous study from our research group that report a deviation from HWE for MR-2G/C in a German population of 218 healthy young subjects (van Leeuwen et al. 2010). As χ^2 tests tend to be inflated in type 1 errors in relatively small samples (Wigginton et al. 2005) this inconsistency will not further be discussed.

The results reported here– cautiously interpreted – may indicate that MR polymorphisms have an impact on cortisol secretion in ambiguous situations containing aspects of contextual novelty. They may exert their effects in different structures of the brain that are interconnected and play a role in the anticipation of a novel situation as a stressor, the susceptibility for social feedback or influence a threshold for the impact of fast reacting systems that are linked with HPA axis function.

5.7 References

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CHAPTER 6

General Discussion

The aim of the present study was to investigate associations between two single nucleotide polymorphisms (SNPs) of the mineralocorticoid receptor (MR) gene and human cognitive functions. The investigation of a comparable association had not been done until the beginning of this project and thus the present investigation was considered as a pilot study. Still, there are only two studies that have investigated the function of the MR in human cognition and one of which was only published after the data of the present study had been analyzed. For this reason, the tests that were used had been chosen based on the findings of rodent studies and the results of Otte et al. (2007), leading to the assumption that MR function is most probably related with executive control functions of the prefrontal cortex. Significant (or tendencies for significant) associations were found in measures of error rates and prefrontal cortical activity for the *Attention Network Task* (ANT) and for the *Wisconsin Card Sorting Test* (WCST). Both are considered to measure executive control while they are characterized by different levels of task complexity. Further, a tendency for a sex specific association between MR polymorphisms and mildly stimulated levels of salivary cortisol in the afternoon could be described. At the end of each chapter the results of the experimental work have been discussed separately and therefore the general discussion will focus on the integration of those results. Furthermore, methodological aspects and limitations of the present study will be discussed. An attempt to draw general conclusions will be made, integrating results of further association studies that address the functions of the two single nucleotide polymorphisms investigated in the present study.

The results of the present study comprising different measures of endocrine parameters and cognitive functions provide a relatively stable base for the interpretation of the functionality of MRI180V (rs5522) and MR-2G/C (rs2070951) in the present sample. The group with the most common genotype (-2GC) was characterized by the lowest error rate in the all three networks of the ANT. Additionally, in the two networks in which we found differences in cortical activity for the ANT, the MR-2G/C heterozygous subjects showed a marked difference in cortical activation between the double cue and the no cue condition in the alertness network. They further were the only group that showed cortical activity in theoretically predictive manner in the conflict network. Furthermore, the -2GC group showed the found sex effect, which consisted of minor changes in salivary cortisol in men during the test session on a descriptive level. In respect to the accuracy measures and assessment of cortical activity for the ANT, the homozygous expression of the C allele of MR-2G/C and the carriage of the MRI180V G allele seemed to be disadvantageous. In all networks, subjects with this genotype committed more errors than subjects of the -2GC and -2GG group. They further showed a reduced difference in cortical activity between the two cue conditions of the alertness network. This indicates that in these two groups, the non-alerting (no cue) condition and the alerting (double cue) condition provoke similar reactions, maybe due to a different

interpretation of the stimulus material than in the other two groups. On a descriptive level, the difference between the no cue condition and the double cue condition in error rates was, like it would be assumed, characterized by a higher error rate for the no cue condition in the -2CC group. The only group in which that difference did not show up was the -2GG group. In subjects carrying this genotype, a relatively low amount of errors was detected and inspection of the descriptive ANT data revealed a comparable error rate for both cue conditions. However, the difference in cortical activity between the cue conditions was lower in the two groups that were characterized by the highest error rates (180V and -2CC) in the alertness network. Likewise, for the conflict network it was shown that the 180V and -2CC group had the highest error rates and were characterized by a particular pattern of cortical activity. While the authors of the ANT suggest that the response to incongruent flankers provokes a higher response conflict (Fan et al. 2003) and this should be reflected by lower relative positivity in EEG measures (Westbrook 2000), the 180V and -2CC group show higher relative positivity during the preparation of a motor response for incongruent flankers. Again, the -2GG group shows a particularity in cortical activation: in subjects with this genotype, activity did not differ between conditions. For both flanker conditions this group exhibited the highest negativity and furthermore is the only group that showed significantly lower positivity under the congruent flanker condition than all remaining three genotype groups. These activity pattern in the -2GG group might indicate that the comparably high level of achievement in terms of accuracy is accompanied by a high level of cerebral activity in the conflict network and an elevated capacity for discrimination between alerting and non-alerting cues in the alertness network.

Accuracy differences between the four MR genotype groups in intradimensional shifts of the WCST showed another picture: here the -2GG group showed the lowest error rate averaged over the first three trials after an intradimensional shift, followed by the 180V group and the -2GC group, and finally, the group with the highest error rates was the -2CC group. Most remarkably, the good performance of the 180V group was distinct from the results of the ANT and furthermore, the higher accuracy of -2CC subjects compared with subjects heterozygous for MR-2G/C. However, the -2GC group showed the steepest learning curve over the course of the first three trials of an intradimensional shift and reached a similar accuracy as -2GG subjects at the third trial. This might indicate a different use of strategies for set shifting by the four MR genotype groups. Intradimensional shifts are relatively easy to detect because they make it impossible to sort cards following the same proxy of one of the two categories as before. There are two possible strategies to resolve this shift detection: either one identifies the shift and directly switch to the other category or one directly detects the shift correctly as intradimensional shift and consequently stays within the same category. The latter strategy might account for the high accuracy in the -2GG group whereas the first

strategy could explain the performance of the -2GC group. An obvious limitation of this study is that the chosen strategy to analyze the event related potentials (ERPs) for set shifting did not allow analyzing intradimensional shifts in terms of cortical activity. The results for frontopolar activity differences during extradimensional set shifting are not representative for intradimensional shifts. Still, the -2GG group showed the lowest positivity in last false and first correct trials of extradimensional shifts and this activity remains stable over the time in first correct trials while the other three genotype groups showed a drop in positivity. Again, this would underpin the assumption that in this genotype group a comparably good task performance is related with higher cortical activity.

In the following section, the study will be discussed addressing prominent questions for genetic association studies. In the last ten years the experience with candidate gene studies for specific phenotypes and genome wide studies has grown a lot. The most prominent challenge of such studies is to allow replication of the results and several suggestions have been made to ameliorate the study design and the report of results (e.g. Ehm et al. 2005; Little et al. 2009). Some of these suggestions and further critical issues will be addressed in order to rule out general implications for the assessment of the association between MR polymorphisms and human cognitive function.

6.1 A priori probability of possible associations

The MR contributes to the regulation of the HPA axis and is expressed in brain structures important for different cognitive functions. To date, most of the research on the relation of the MR and cognitive function has been done in rodents. Studies have focused on its role for cognition and often addressed the question of the role of the MR for the regulation of the hypothalamus-pituitary-adrenal (HPA) axis. Affected behaviors in rodents have been described for different methodological approaches including tissue specific gene knock-out or overexpression in cerebral tissues to avoid side effects of an altered aldosterone signaling in peripheral tissues. It can be considered as promising that the results of studies, using theses techniques, suggest distinct behavioral changes, related to the affected tissues, and sometimes report of an affected HPA axis (re-) activity (Berger et al. 2006; Lai et al. 2007; Rozeboom et al. 2007; Ferguson and Sapolsky 2008; Mitra et al. 2009). Certainly, the translation from rodent into human behavior itself would be a somehow risky approach to justify the choice of a distinct cognitive domain for a genetic association study in humans. On the other hand, results of human genetic studies already indicated the functionality of MR polymorphisms in HPA axis reactivity (DeRijk et al. 2006) and a possible role in the development of major depression (Kuningas et al. 2007). Furthermore, the functionality of MRI180V for altered transcriptional activity of the MR had been shown *in vitro* (DeRijk et al. 2006). Altogether, these findings seemed to be a good rationale to investigate the

association between polymorphisms in the MR gene and executive cognitive functions in humans.

Only very recently two additional studies have been published about the functionality of the two polymorphisms (rs5522 – MRI180V, rs2070951 – MR-2G/C) assessed in the present study, in respect to HPA axis function. The findings of both of these investigations (Bouma et al. 2010; Muhtz et al. 2010) are contrary to those of our research group (DeRijk et al. 2006; van Leeuwen et al. 2010), which will later be discussed in detail. The only investigation of the association between MR polymorphisms and a specific cognitive endophenotype in humans has been published only very recently too and reports of a promising relation between MR polymorphisms, stress and reward learning. As reward learning has been considered as a prefrontal function, this study can contribute to a fruitful discussion of our results.

6.2 Phenotyping and methods

The quality of phenotyping is essential for obtaining valid results when carrying out genetic association studies. The classical approach for genetic association studies is the comparison between cases and controls, i.e. testing if a distinct allele is more common in people affected with a psychiatric disease than in a healthy population. Still, psychiatric diseases are characterized by high phenotypic heterogeneity and overlapping neurobiological symptoms. Therefore, exact definitions of endophenotypes are needed to increase the likelihood to detect associations with conscientiously chosen candidate genes. In the case of polymorphisms in the MR gene, the phenotype most commonly studied was pseudohypoaldosteronism type 1 and no study had been carried out until the beginning of the present investigation that addressed the association between MR polymorphisms and cognition in humans. Depression as a stress related psychiatric disorder has been investigated in relation to MRI180V and MR-2G/C and an influence of the G allele of MRI180V has been found associated with the development of depressive symptoms in a longitudinal study of old adults. Associations of MR polymorphisms with cognitive tasks to specifically detect impairments in attention, processing speed, immediate or delayed memory have not been described for this population (Kuningas et al. 2007). For the operationalization of the present study these results were nonetheless very helpful because they might indicate cognitive domains in which the MR might not be involved and that consequently can be excluded from further investigation.

The neuroendocrine stress system is a probable candidate to underlie more than one psychiatric illness since deregulations of the HPA axis have been described for depression, anxiety, post traumatic stress disorder, schizophrenia or bipolar disorder (see Chapter 2.1.1). Therefore, a careful modeling of stress related endophenotypes seems important to unravel the interrelations between stress neurocircuitry and other neurotransmitter systems. The

differentiation between MR and glucocorticoid receptor (GR) affected cognitive domains is one important step in this direction. In the present study this attempt was made by defining MR related cognition mostly on the base of results from rodent studies. We focused on executive cognitive control and used EEG as a sensitive instrument to detect subtle changes in cortical activity. One advantage of the EEG technique, compared to imaging methods like positron emission tomography or magnet resonance tomography, is the high temporal resolution of EEG. Albeit its low spatial resolution, it is an appropriate instrument to assess activity of the prefrontal cortex which is essential for executive cognitive control. Further, the tests that were applied to measure executive cognitive functions are sensitive to prefrontal activity and assess different levels of complexity of executive control. The measurement of the cortisol awakening rise (CAR) plus the short daily profile of salivary cortisol is a broad but economic measure of HPA axis activity. Together with the assessment of salivary cortisol during the afternoon test session, we intended to cover possible influences of the MR on cortisol secretion.

6.3 Composition and size of the sample

Because of the exploratory approach of the study and the aim to avoid confounding effects we chose to recruit a sample of young healthy participants. They were all students or employees of the Trier University. Unintentional population stratification is unlikely, however not impossible, as most of the Trier students come from various parts from Germany. Ethnicity had been controlled for and all of the participants came from European countries, most of them were German. We only included non-smokers and subjects without a history of physical or psychological disease as well as subjects that were under chronic or acute medication. Participants of the main study were in the age range from 18 to 38 years. The genotyping was accomplished before participants were re-invited for the main investigation. This was done to avoid unequal group sizes for statistical reasons due to the rare appearance of the MRI180V G allele. We did not carry out a haplotype analysis but in all samples investigated for MR polymorphisms in our research group, three haplotypes have been identified that account for 96% of all populations (personal communication Roel de Rijk). These three haplotypes comprise all allele combinations of MR-2G/C and MRI180V that were found in our sample. A clear limitation for the interpretation of our results is that the 180V group in our sample is heterogeneous for MR-2G/C and is composed of subjects heterozygous for MR-2G/C or subjects homozygous for the C allele. Homozygous carriers of the MR-2G/C G allele were not found in combination with the rare G allele of MRI180V. In all of the four groups investigated in the main study, both sexes were about equally distributed. Only women that took oral contraceptives, not containing drospirenone as potential MR antagonist (Oelkers 2004), were invited for the main study.

A further important limitation is the small size of our sample for a genetic association study and for that reason the analysis of salivary cortisol can only be regarded as exploratory. However, the sample size can be considered as sufficiently big for the cognitive testing. This is because of the frequent repetition of the test's conditions that lead to increased statistical power to detect even small effect sizes by investigating relatively small samples. Furthermore, the paradigms used are valid cognitive measures, sensitive for executive functions driven by the prefrontal cortex.

6.4 Statistical analysis

All analyses were calculated in the form of one-way analyses of variance (ANOVAs) for repeated measures with 'genotype' as between subject factor for the analysis of reaction times and accuracy as well as for ERP components. For cortisol analyses, we additionally integrated sex as between subject factor because it has repeatedly shown that the sex of the subjects is associated with HPA axis outcome (see Kudielka and Kirschbaum 2005 for a review) and sex effects have been shown in another association study addressing the functionality of GR single nucleotide polymorphisms (SNPs) in HPA axis reactivity (Kumsta et al. 2007). Careful definition of phenotypes normally enhances the power of genetic association studies and therefore we expect that the results of the present investigation provide a solid base for replication. Nevertheless, multiple testing may lead to an emergence of false positive results. The p-values for effects in the different ANOVAs are not corrected for multiple testing and should hence be interpreted with caution. On the other hand, we applied the *Dunn's Procedure of Multiple Comparisons* in the case of (marginally) significant interactions. This test has not forcedly been considered as pure post-hoc tests and comparisons exceeding the critical difference can be considered as significant on an α-level that has been corrected for the amount of comparisons made within the respective ANOVA.

6.5 Gene-gene interactions

We certainly can not exclude gene-gene interactions that might modulate the association between MR SNPs and executive functions. Quite the contrary, it is highly probable that human executive functions are modulated by more than one neuroendocrine or neurotransmitter system. Genetic association with measures of the *Attention Network Task* (ANT) have been shown for the dopaminergic system (Fossella et al. 2002; Fan et al. 2003) and for a coding SNP in the *tryptophan hydroxylase 2* gene, indicating a role of serotonine in executive control (Reuter et al. 2007). Some studies have reported about associations between a non-synonymous coding SNP in the gene coding for Catechol-*O*-methyltransferase (COMT) and parameters of the *Wisconsin Card Sorting Test* (WCST) (Egan et al. 2001; Rosa et al. 2004; Minzenberg et al. 2006). This indicates a role of

dopaminergic or noradrenergic circuits in cognitive set shifting. It would be interesting to investigate the influence of different neurotransmitter systems, represented by polymorphisms of genes that code for molecules important for the functioning of these transmitter systems. Problems of studies addressing the interplay of whole systems with the help of genetic polymorphisms are above all the investigation of many different genotype groups which will always have unequal sizes, the exact phenotyping, and the use of methods that have high temporal and spatial resolution to disentangle the interplay of different neurobiological systems.

6.6 Gene-environment interactions

The endocrine stress system reacts sensitive to environmental changes. For example, prenatal factors, post-natal child-parent interactions and socioeconomic status have been identified to modulate HPA axis regulation (as reviewed in Lupien et al. 2009; McEwen and Gianaros 2010). In squirrel monkeys it has been shown that chronic social stress in adult animals can diminish MR expression in the prefrontal cortex, whereas for early life stress GR expression decreases in this region (Patel et al. 2008). It was not controlled for environmental factors like early life experiences or recent prolonged stress periods and therefore possible interactions of the participant's genotype and their environment cannot be excluded.

An important assumption for the interpretation of our results is that the investigated SNPs are representative for the functionality of the MR protein or, in the case of MR-2G/C eventually the amount of the protein. Haplotype analyses of large cohorts which were carried out in our research group revealed that three major haplotypes for the two SNPs are representative for a region comprising exon 2 and extending into the promoter. In this region, further SNPs and a two basepair insertion/deletion polymorphism are located (Klok, unpublished data) and until now, nothing is known about their *in vitro* functionality. The two SNPs investigated in our study have been shown to alter the transcriptional activity of the MR as a transcription factor. In the case of MRI180V this means that the minor allele decreases transcriptional capacity specifically for cortisol as a ligand, compared to aldosterone (DeRijk et al. 2006). Very recently, the MR-2G/C has been tested for *in vitro* functionality in more detail. The G variant attenuates transcriptional activity for cortisol, dexamethasone and aldosterone as a ligand and further decreases the expression of the protein, which is more pronounced in combination with exon 1α than for exon 1β. The mRNA expression was not modified by MR-2G/C and that indicates that expression changes happen on a translational level (van Leeuwen et al. 2010; van Leeuwen et al. 2010). Still, the MR gene (*NR3C2*) is far from being well understood. The knowledge about expression of different variants of the protein or

mRNA or about their functionality is much smaller than for GR. The variety of in vitro tested cell lines is restricted to only few and non-human cells, and further no cerebral cell line has been tested until now. The cellular environment, however, plays a crucial role for the function of MR as it defines the transcription limiting factors of the protein itself by the different availability of relevant transcription factors or ligands that enable the transcriptional activity of the MR (see Chapter 2.5 for detailed description of influencing factors). Further, the transcriptional activity might be influenced by the respective promoter constructs used *in vitro* and possibly does not reflect *in vivo* transcriptional functionality.

Very recently, the MR-2G/C polymorphism has been found to be associated with higher levels in overall cortisol, and specifically increased cortisol levels in the morning in subjects homozygous for the G allele (Muhtz et al. 2010). This is in contrast to the results of our research group that found both, MRI180V and MR-2G/C unrelated with unchallenged morning cortisol levels and report of a sex specific association with morning cortisol levels after oral administration of 0.25 mg cortisol the evening before (van Leeuwen et al. 2010). A possible explanation for these results could be the different age of the investigated populations. The former study assessed participants in the age range from 30 to 70 years whereas the latter study investigated young adults between 18 and 36 years. Given that the MR-2G/C alters transcriptional activity of the MR and thereby influences downstream signaling constantly, the HPA axis and related systems might adapt over time and develop counter regulatory mechanisms over time. A further study of our research group reports of a sex specific association of MR-2G/C with morning cortisol levels in depressive participants using selective serotonin reuptake inhibitors. Men and women homozygous for the G allele showed the highest morning cortisol levels but whereas in women homozygous for the C allele the awakening rise was completely blunted, this genotype in men had higher levels of morning cortisol than MR-2G/C heterozygotes and men homozygous for the G allele (Klok et al.). To further complicating the picture, another recent study reports no association of either MRI180V or MR-2G/C with stimulated cortisol levels or heart rate in a psychosocial stress task in a large sample of adolescents (15-17 years) (Bouma et al. 2010). Not considering the age difference between the populations under investigation, this finding is contrary to the finding of our research group that carriers of the G allele have increased salivary cortisol responses as well as an increased heart rate after participating to the *Trier Social Stress Test* (TSST) (DeRijk et al. 2006). Again it can be hypothesized that the age difference promotes the failure of replication of the previous results. Additionally, the stress protocols used differ in the amount of social evaluation and increases in heart rate and salivary cortisol were lower in adolescents compared to young adults. The authors of the former study argue that the lack of association might be due to the correction of multiple testing that they applied to their analyses. This seems unlikely considering the very low p values reported for the latter

study and the fact that in the latter study only three models (heart rate, salivary cortisol, and plasma cortisol) were calculated for a sample of men only. The researchers of the former study instead applied distinct models for two different measures (salivary cortisol and heart rate) as well as for three subsamples (boys, girls using oral contraceptives, and girls not using oral contraceptives). Consequently, a correction for multiple testing was essential for the study in adolescents and can rather be seen as a correction for possible interdependence between the parameters assessed in DeRijks' study. Nonetheless, the correction for multiple testing is often neglected in genetic association studies, which could be a major problem for the replication of existing results.

Finally, the MR-2G/C has been found associated with the renin-alosterone system. In healthy subjects under a diet containing high levels of sodium and reduced levels of potassium as well as in mildly hypertensive subjects, plasma rennin and aldosterone levels were decreased in subjects homozygous for the C allele. Men homozygous for the C allele had lower systolic blood pressure in the mild hypertensive group and in a large cohort of subjects with current or remitted anxiety and/or depressive disorders (van Leeuwen et al. 2010). In the same cohort of mildly hypertensive subjects as assessed in this study, no association with MRI180V in any of the parameters mentioned above had been found (DeRijk et al. 2006). The association between MR polymorphisms and blood pressure in depressed and anxious male participants might lead to interesting hypotheses for the future investigation of the role of MR for psychiatric disorders.

The interpretation of our data compared to the most recent findings about the function of the MR for endocrine and physiological measures or cognition remains difficult. Our participants were of about the same age as the sample of de Rijk et al. (2006), Bogdan et al. (2010), and van Leeuwen et al. (2010). If we would assume that the test situation could be interpreted as a mild stressor, the finding that male 180V carrier had an elevated level of salivary cortisol would underpin the higher stress reactivity in this group found by de Rijk et al. (2006). The only study assessing the MR-2G/C polymorphism in association with a challenged HPA axis was carried out in adolescents and in this study, no significant differences were found between the genotypes (Bouma et al. 2010). In this study, a relatively large cohort of 473 adolescents had been investigated and so its results are far less explorative than ours. Howsoever, adolescence is characterized by important changes in sex steroids that influence HPA axis activity (reviewed in Lupien et al. 2009). It has been suggested that adolescence is associated with elevated basal and stress-induced activity of the HPA axis (Gunnar et al. 2009). Further, levels of GR in the prefrontal cortex are higher than during childhood and later in life (Perlman et al. 2007). This might indicate an adaption of prefrontal structures on higher cortisol levels and conclusively during adolescence the role of MR in HPA axis regulation might be distinct from later periods in life.

The role of the MR in HPA axis reactivity is highly interesting and may closely be linked to stress related cognition. If our results for the salivary cortisol data would be reliable, it would be interesting to investigate if the minor allele of MRI180V is associated with stress anticipation, whereas the C allele of MR-2G/C might rather be important for the interpretation of potentially stress inducing (social) cues. Subjective psychological responses to stress (anxiety) and the secretion of cortisol only correlate if they are analyzed in a time lagged design (Schlotz et al. 2008). While psychological stress responses can change within dynamically seconds, the endocrine stress system reacts about 15-20 minutes after the onset of a stressor (Kudielka et al. 2009). Further, multiple physiological systems may contribute to acute psychological stress responses and possibly interact with HPA axis related parameters. It will therefore be helpful to concentrate on MR related cognitive functions and to find hypotheses how distinct cognitive operations can predict acute cortisol secretion. Our results suggest a role of the MR in prefrontal cortex-driven executive control, whereas Bogdan et al. (2010) focus on reward learning as depression-related cognitive pattern. Both, reward learning and executive functions are linked to activity of the prefrontal cortex (Wallis and Kennerley 2010), and consequently it can be assumed that the MR plays a role in prefrontally driven cognition.

To date, the role of MR for the development of mental disorders remains to be elucidated and has only been addressed in one study (Kuningas et al. 2007). On the other hand, dysregulated HPA axis activity has been found in association with multiple psychiatric disorders. As cortisol binding receptor in central structures that is associated with distinct cognitive operations that are affected in patients suffering from psychiatric diseases, the MR can hence be considered as a good candidate gene for the detection of endophenotypes that lead to increased vulnerability for mental disorders.

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CHAPTER 7

Outlook

In the last decade, a lot of progress has been made in the research field of behavioral genetics. Huge cohorts of patients, suffering from psychiatric disorders as well as healthy controls have been screened genome wide, in order to detect candidate polymorphisms that differ in frequency between patients and controls. Further, imaging genomics have provided evidence that it is possible to link even single polymorphisms to alternations in activity in distinct cerebral tissues. Additionally, analyses of epigenetic markers like methylated cytosines or changed conformation of histones can be carried out (epi-) genome wide and have become increasingly faster and less cost extensive. Still, it has turned out that the development of methods that generate more and more data requires the development of new statistic strategies to identify gene variants, contributing to the vulnerability of developing complex diseases. Hypothesis driven candidate gene association studies normally report of very low effect sizes indicating that polymorphisms in a single gene account for a very low percentage of explained variance in large groups of patients with common diseases. Statistic modeling of genome wide association studies (GWAS) is confronted with the problem that it has to be accounted for multiple testing and with α-error correction for up to 5.5 billion polymorphisms. Even if GWAS detect biological meaningful genetic variants, these only account for minor effect sizes and most of them are common variants. Furthermore, to find out variants accounting for minimal percentages of explained variance of a psychiatric disease, carrying out GWAS necessitate sample sizes of several thousands of cases and controls. Samples of this size on the other hand almost guarantee a high level of heterogeneity for the phenotype under investigation and consequently, mostly common gene variants that may account for a general vulnerability for a psychiatric disease are detected. Rare variants may rather be characteristic for distinct endophenotypes, and therefore would be good candidates to understand the biological mechanisms underlying mental illness. To find such variants using a GWAS' approach would require the investigation of still bigger samples. Further, to relate rare variants with specific behavioral or cognitive subclasses, theory-based modeling of such subclasses would still be needed. Another approach would be to restrict the amount of investigated polymorphisms and to choose candidate genes for disease-related brain systems *a priori*. Then sample sizes could be decreased and efficient phenotyping could more easily be warranted. A further step in the operationalization of genetic association studies could be structural path modeling of assumed association strengths' with certain phenotypes and interrelations between different genetic variants. The base for such an investigation would be provided by the results of studies assessing the same phenotype in relation with different genetic polymorphisms and/or those assessing the association of a genetic variant with different phenotypes.

In the following, some issues will be addressed that should be considered when investigating the association of the MR in human cognitive function. Simultaneously testing large regions

of the human genome and the information about variation, provided by comprehensive data bases enables the development of new study designs. To obtain valid and interpretable results, some influencing factors should be taken to account, that have shown to be related to HPA axis function and are probable candidates to increasing the percentage of explained variance.

7.1 HPA axis related sexual dimorphism

A general finding in stress research is that men and women differ markedly in parameters of HPA axis activity and reactivity. Furthermore, women not taking hormonal contraceptives show a different HPA axis regulation between the different phases of their menstrual cycle (reviewed in Kudielka et al. 2009). It is well known that HPA axis activity is heavily dependent of the sex of the subjects and that sex related hormones like testosterone, estrogen or oxytocin are linked with HPA axis activity but also account for sex differences in cognitive processes that may be crucial in stressful situations (van Honk et al. 2004; Hermans et al. 2006; Lissek et al. 2007; Wirth and Schultheiss 2007; van den Bos et al. 2009). Hippocampal volume has been shown to be associated with levels of circulating estrogen (Lord et al. 2008) and sex steroids are associated with brain development during puberty (Neufang et al. 2009; Peper et al. 2009).

On a molecular base several pathways have been identified by which a close interaction between glucocorticoids (GCs) and sex steroids may be modulated. The MR is a 'promiscous' receptor that binds to aldosterone, cortisol, and corticosterone with similar affinity (Gomez-Sanchez 2009). Further, it can bind to progesterone which has antagonizing effects on MR function (Yang and Young 2009) and in rats it has been shown that progesterone is related to MR expression regulation (Castren et al. 1995). In the rat brain, sex specific changes in MR and GR mRNA expression have been described after application of an acute stressor (Karandrea et al. 2002) and in humans, sex specific MR and GR expression has been found in patients with focal epilepsy (Watzka et al. 2000). The molecular mechanisms of steroid hormone interactions are not well understood and the sexual dimorphism in distinct cognitive processes remains to be further elucidated. To gain more insight in these mechanisms, *in vitro* studies addressing the functionality of genetic variants in various human tissues under systematic investigation of different ligands and heterodimerization would be helpful. Additionally, the simultaneous assessment of polymorphisms in potentially interacting systems in larger cohorts would help to disentangle the interplay of steroid hormones. Anyway, it is possible that the etiology of stress-related psychological disorders may vary considerably between the sexes and therefore the influence of sex steroids should be considered whenever the relation between stress and cognition is assessed.

7.2 Environmental influences and epigenetics

Influences of life events have a long tradition in the exploration of psychiatric disorders and the 'nature or nurture' debate has separated the psychological from the biological modeling of mental illnesses for a long time. To date, the debate seems anachronistic as the interaction between genetic variability and environmental factors for the development of mental diseases has been shown in several illustrative studies (Caspi et al. 2002; Caspi et al. 2003; Caspi et al. 2005; Brookes et al. 2006; Fowler et al. 2009). Furthermore, in rat models a molecular mechanism has been described that links early maternal care to stress reactivity later in life. This mechanisms comprises the methylation of a CpG dinucleotide in the promoter region of the GR, which results in decreased gene expression because the methyl group blocks the access of a distinct transcription factor to the regulatory region (Weaver et al. 2004). The methylation of cytosines in the promoter region of genes since is discussed as a general biological mechanism that may be sensitive for distinct environmental influences. The question of how persistent these epigenetic modifications are still is debated. It has been proposed that there may be critical periods for persistent cytosine methylation changes but it is possible that the methylation of cytosines is a highly adaptive and flexible mechanism for the organism to react to environmental influences. Interestingly, a recent review about sex specific cytosine methylation of the estradiol- and progesterone receptor suggests that methylation can change across the lifespan and that, contrarily to earlier assumptions it is not forcedly related to gene expression. Nevertheless the authors argue that exposure to estradiol in neonatal rats can lead to epigenetic alternations and that these changes can mediate estradiol-induced sexual differentiation of the rat brain in a persistent manner (Nugent et al. 2010). The more flexible epigenetic regulation changes are over life time the more it will be difficult to control for possibly affecting life events or other environmental factors like, for example, nutrition. To approach to address influences of environmental factors prospective longitudinal studies of large cohorts would be important, although cost intensive.

7.3 Imaging genomics - methodology

Imaging genomics is another field in neuroscience that has gained a lot of interest for integrating genetics with brain imaging techniques (Winterer et al. 2005). Under meticulous control for non-genetic influence factors, distinct neural circuits are assessed in response to specific auditory, visual, and cognitive or emotional stimuli to investigate the functional impact of genetic variants. Since 2002, when the first paper linking the function of genetic variants of the serotonin transporter gene to the activity of the amygdala during the presentation of fearful and threatening faces (Hariri et al. 2002), a lot of studies have been published integrating imaging genomics. Most of these studies used functional magnet

resonance tomography as method to detect activity differences in experimental groups, characterized by their genotype for a distinct gene. This method allows high spatial resolution that can help to identify specific brain regions that are related to the functionality of the genetic variants. However, the disadvantage of fMRI is the poor temporal resolution of brain activity images, which may hinder the exact description of brain processes that are related to distinct neural systems and that are influenced by specific genes. During the perception, integration and interpretation of stimuli, neuronal circuits are activated in parallel and transiently overlap in activity and so a low time resolving technique may misestimate the association of a distinct gene and stimulus related activity of theoretically mapping brain area. The method with the highest temporal resolution to assess cortical activity still is electroencephalography (EEG). In the past years this method has regained attention as method to investigate the function of genetic polymorphisms and this year a first study using a genome wide association approach to assess candidate genes for the heritability of EEG activity has been published (Hodgkinson et al.). Developing mathematic modeling of EEG data today allows the identification of source generators and consequently the comparison with results from other imaging techniques (e.g. Grech et al. 2008). A further developing method has the potential to help on the investigation of stress-related cognitive processes is the single photon emission computed tomography (SPECT). This technique would allow assessing the distribution of MR and GR in the living human brain, given that radioisotopes will be developed that specifically bind to the receptors.

7.4 Tissue specifity

As described in Chapter 2, the MR binds to either cortisol or aldosterone, depending on the cellular environment. The MR-2G/C polymorphism has been shown to be associated with stress-related measures but has also been found to be related to alternations in the rennin-angiotensin-aldosterone system. Functional characterization *in vitro* revealed different efficacy for MR translation for the two alleles, specifically in combination with the alternative exon 1α compared to exon 1β, depending on the cell line tested (van Leeuwen et al. 2010; van Leeuwen et al. 2010). These findings already suggest a tissue specific regulation of the transcription and/or translation of the MR which is highly plausible bearing in mind the functional diversity of the receptor. Furthermore, albeit the earlier assumption that MR expression is more restricted than expression of the GR, the MR now is considered as ubiquitous transcription factor (Viengchareun et al. 2007). MR and GR display a marked homology (Arriza et al. 1987) and evolved from a common ancestor (Baker et al. 2007; Ortlund et al. 2007). Nonetheless, the 5' untranslated region of the GR carries nine alternative first exons, accounting for eleven alternative splice variants that are characterized by a highly tissue dependent expression (Turner et al. 2006; Presul et al. 2007) while for the

MR, only two alternative fist exons are known (Yang and Young 2009). As already mentioned in regard to sex differences in HPA axis activity, it would be recommended to further analyze the expression of different MR transcripts in various tissues, as it seems very probable that the tissue specific regulation of the MR is considerably similar to the regulation of the GR.

7.5 References

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbst verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Anne Molitor