Mineralocorticoid receptor gene variants : implications for stress, blood pressure and personality

Leeuwen, N. van

MINERALOCORTICOID RECEPTOR GENE VARIANTS

Implications for stress, blood pressure and personality

Nienke van Leeuwen

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Contents

1

General Introduction

Contents

Everyone occasionally feels "depressed" but these feelings are usually temporary and pass within a couple of days. This is not what occurs when a person has a depressive disorder (e.g. major depression). The symptoms persist for at least two weeks and interfere with a person's ability to work, study, sleep, eat and enjoy pleasurable activities. Depressive disorders are a leading cause of disability worldwide (Murray and Lopez, 1997). Major depression is therefore a serious illness and unfortunately a very common illness; over 15% of the Dutch population experiences a depression during their life (Bijl et al., 1998).

A central question is "why do some persons get depressed while other persons thrive under similar adverse conditions?" A follow up question in line with this notion is "what are the biological mechanisms leading to a depression?" Unfortunately, we do not know the answers to these questions yet. Many studies have been performed and it has been shown that a stressful event often precedes depression; people who have experienced severe chronic stress develop a depression more easily than individuals who have not experienced stress. Therefore it has been hypothesized that the stress system is part of a biological mechanism involved in the development of the disease.

However, after experiencing the same stressful life events, only some subjects develop clinical symptoms of depression. This indicates that these individuals are more vulnerable suggesting a genetic component or developmental disturbances. Indeed, the involvement of a genetic factor was shown by studying families. It is very clear that depression runs in families; however members of a family not only share their genetic background but also environmental factors, like financial status or the death of a family member. However, after correction for the environmental factors in these families, it was still found that there is a genetic part (estimated at ~ 40%) causing vulnerability to the disease. The model proposed to explain this vulnerability is the so called "three hit" model: genetic variation in interaction with early life experiences enhances vulnerability to a challenge in later life which precipitates depression.

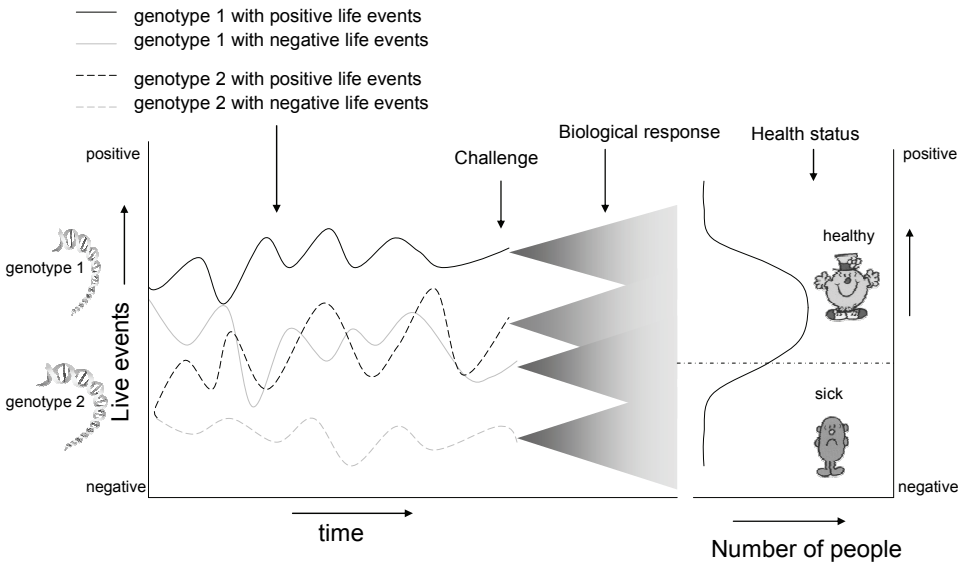


Figure 1. Schematic overview of the three hit model. Genetic variation (first hit) in interaction with life experiences (second hit) influences the response of brain and body following a challenge (third hit) later in life.

The goal of this thesis is to elucidate genetic variation underlying stress-induced vulnerability to depression. For this purpose genetic variants of one of the receptors for the stress hormone cortisol, the mineralocorticoid receptor (MR) were studied. In the introduction of this thesis the Hypothalamus Pituitary Adrenal (HPA) axis, its end product cortisol and the different types of receptors are described first. Then, methods for testing HPA axis functioning are discussed and finally the current state of art in understanding the role of HPA axis dysregulation in mental health is evaluated. The second part of the introduction describes genetic structures and consequences of genetic variation of the corticosteroid receptors. The introduction is concluded with the scope, objective and outline of this thesis

1. Stress and the Hypothalamus Pituitary Adrenal (HPA) axis

A stressor or 'stress' is any physical or psychological threat to homeostasis, which is the equilibrium in life processes. The stress response is the spectrum of physiological and behavioural adaptations to restore homeostasis. Physical stressors like an infection, tissue damage or pain activate aminergic neurons in the brainstem while psychological stressors require processing of the stressful information in limbic brain areas such as the amygdala, hippocampus and prefrontal cortex. Afferents from the brain stem and the limbic regions innervate the paraventricular nucleus in the hypothalamus which organizes the sympathetic, neuroendocrine and behavioural response to the stressor.

The sympathetic activation leads to the release of adrenalin from the adrenal medulla into the bloodstream. Adrenalin increases heart rate and cardiac output and this in turn will lead to activation of skeletal muscles, elevation of blood glucose levels and suppression of the digestive and reproductive systems. The neuroendocrine response proceedes mainly via the HPA axis (Figure 2). Neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin releasing hormone (CRH) and its co-secretagogue arginine vasopressin (AVP) in the portal vessel system. These peptides stimulate the anterior pituitary to generate adrenocorticotropin hormone (ACTH) from the pro-opiomelanocortin (POMC) precursor for release in the circulation and this in turn stimulates the adrenal glands to produce and secrete glucocorticoids. In human the main glucocorticoid is cortisol while in rodents this is corticosterone. The glucocorticoids feed back on hypothalamic CRH neurons and pituitary corticotrophs to normalize stress-induced HPA axis activation. The limbic structures are also a prominent target of glucocorticoids, where the hormones modulate ongoing information processing related to the initial stressor that led to the production of the hormone. The most potent psychological stressors are conditions of uncertainty, no control and no information to predict upcoming events. During processing in the limbic brain, such severe psychological stressors lead during processing in the limbic brain to strong emotional reactions and a profound prolonged activation of the HPA axis.

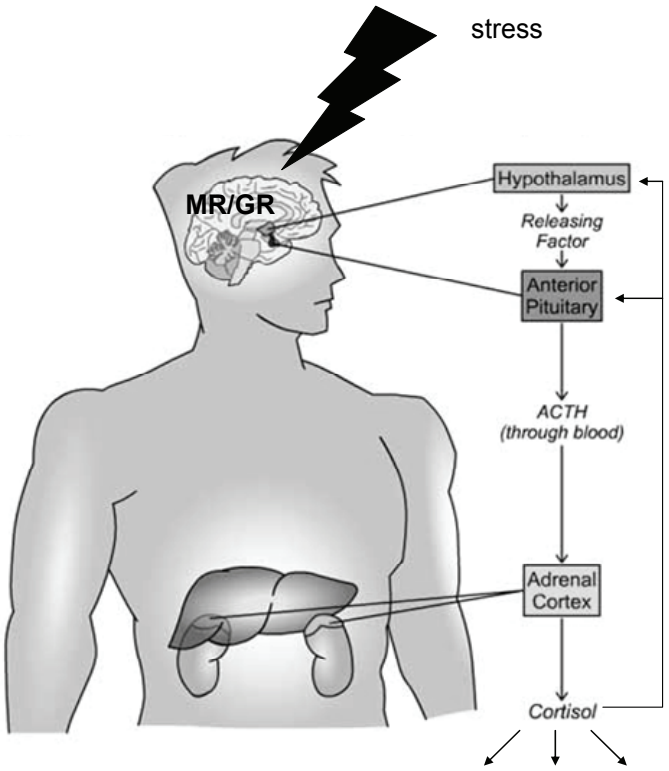


Figure 2. Schematic representation of the HPA-axis

In addition to the stress responsive mode, HPA axis activity displays a circadian rhythm. Highest levels of ACTH and cortisol are observed in the morning prior to the active period followed by a continuous decline resulting in low basal cortisol levels in the afternoon and evening (Schmidt-Reinwald et al., 1999; Linkowski et al., 1993; Spath-Schwalbe et al., 1991). The circadian secretion pattern of cortisol is based on hourly pulses which have in man highest amplitude at the circadian peak in the morning (Lightman et al., 2008; Conway-Campbell et al., 2007). Superimposed on this rhythmic pattern is a one hour lasting surge in cortisol induced by awakening, the so called cortisol awakening response (CAR). There is a small anticipatory increase in cortisol around lunch (Wilhelm et al., 2007; Wüst et al., 2001; Wüst et al., 2000). The stress response is also superimposed on basal rhythmicity and it appeared that the magnitude of stress-induced HPA activation was much higher on the ascending than the descending phase of the ultradian pulse (Sarabdjitsingh et al., 2010)

Cortisol exerts a dual action in the stress response. Cortisol promotes the initial reaction to the challenge and subsequently prevents this initial reaction from overshooting and becoming damaging itself. The latter action is best known. Cortisol and its potent synthetic glucocorticoid analogs such as dexamethasone and prednisone have a potent anti-inflammatory and immunosuppressive action. Also energy storage and mobilization is a critical function of cortisol (McEwen et al., 1979). During stress cortisol provides the body with energy through gluconeogenesis, the process of converting amino acids into readily useable glucose in the liver. Additionally, fat from storage depots is reallocated to fat cell deposits deep in the abdomen (Epel et al., 2000) while directing adipocytes to develop into mature fat cells (Tomlinson et al., 2002). During rest cortisol promotes glycogen production for storage of energy (Fig. 2).

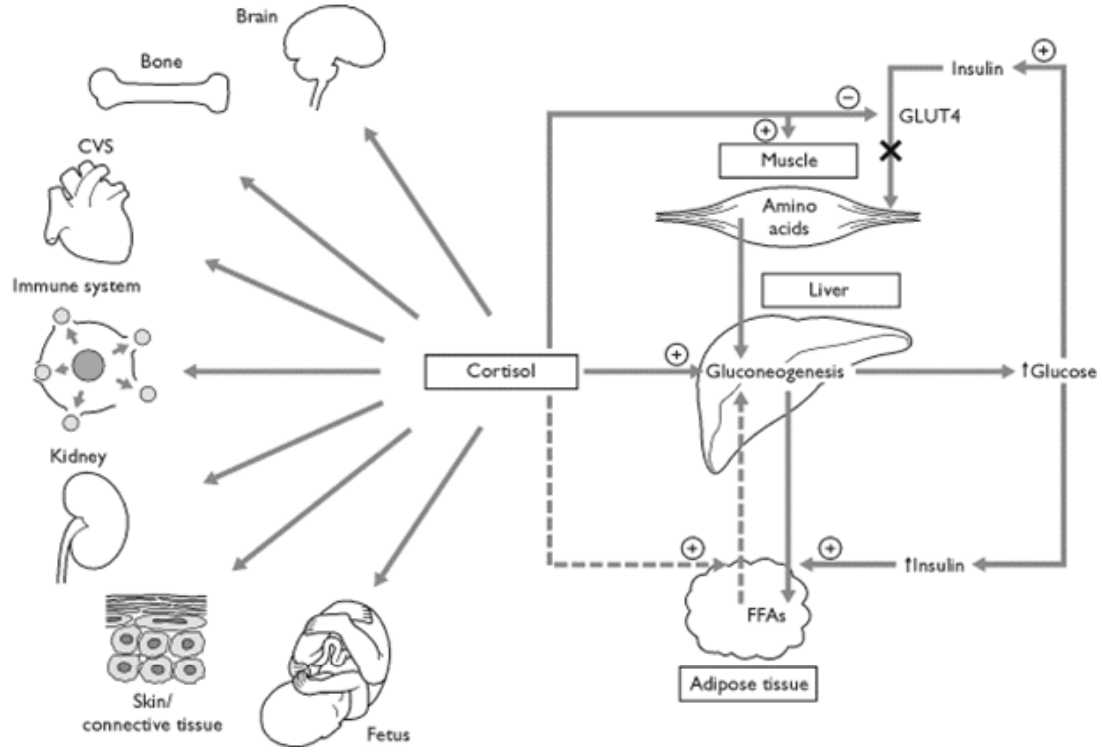


Figure 3. Schematic overview of cortisol target tissues.

Although the action of cortisol is protective in the context of a challenge, it can turn into a harmful signal if for example exposure is either prolonged or inadequate. Prolonged cortisol exposure can lead to suppression of the immune system, visceral obesity and muscle breakdown since amino acids are used for glucose production instead of muscle formation. The consequences are described in more detail in paragraph 1.3.

In the control of basal rhythmic and stress-induced processes in the brain cortisol operates via two types of receptors: mineralocorticoid (MR) and glucocorticoid receptors (GR), which are best known as nuclear receptors regulating gene transcription. MR binds cortisol with high affinity. As a consequence MR always remains substantially occupied with cortisol throughout the ultradian and circadian cycle; this receptor is predominantly expressed in limbic structures. The widely distributed lower affinity GR only becomes occupied at ultradian peaks and after stress. Besides these nuclear receptors recent evidence has also identified MR and GR in the membrane of cells which responds to high stress levels of hormone.

The two receptor types mediate the action of cortisol on the different phases of the processing of stressful information. While the nuclear MR is primarily involved in the maintenance of the integrity and stability of the stress circuitry, its membrane variant is implicated in the onset and progression of the psychological stress response. The GR mediated action by cortisol is concerned with termination of the stress response, the recovery from the stressful challenge and the facilitation

of behavioural adaptation in preparation for future challenges. Accordingly, the balance between MR and GR mediated actions plays an important role in the processing of stressful information (de Kloet et al., 1998). Changes in the balance of MR:GR mediated actions affect HPA axis reactivity and behavioural adaptation: we hypothesize therefore that the balance in MR:GR mediated actions is important for vulnerability and resilience to stress-related mental disorders.

1.1. corticosteroid receptors

In the classical view MR and GR are intracellular receptors which reside in the cytosol in the absence of ligand. After binding a ligand at the ligand binding domain (LBD) the receptors form dimers and translocate to the nucleus where they regulate the transcription of specific genes by binding to the DNA with the DNA binding domain of the receptor (DBD). Depending on the target gene this can be a positive or negative regulation. Additional levels of control have been identified contributing to the diverse effects of glucocorticoids. For example, translocation of the receptors is modulated by chaperone proteins, the conformation of the receptor can be modified by posttranslational changes, expression of coactivators (e.g. SRC1) or corepressors (NcoR, SMRT) while modifications of the DNA structure (methylation, acetylation) can confer additional specificity (Pascual-Le Tallec and Lombes, 2005) (fig 4). Finally, gene-variation in MR and GR can contribute to their function. In paragraph 2.2 of this chapter the gene structure and consequences of genetic MR and GR variation are summarized.

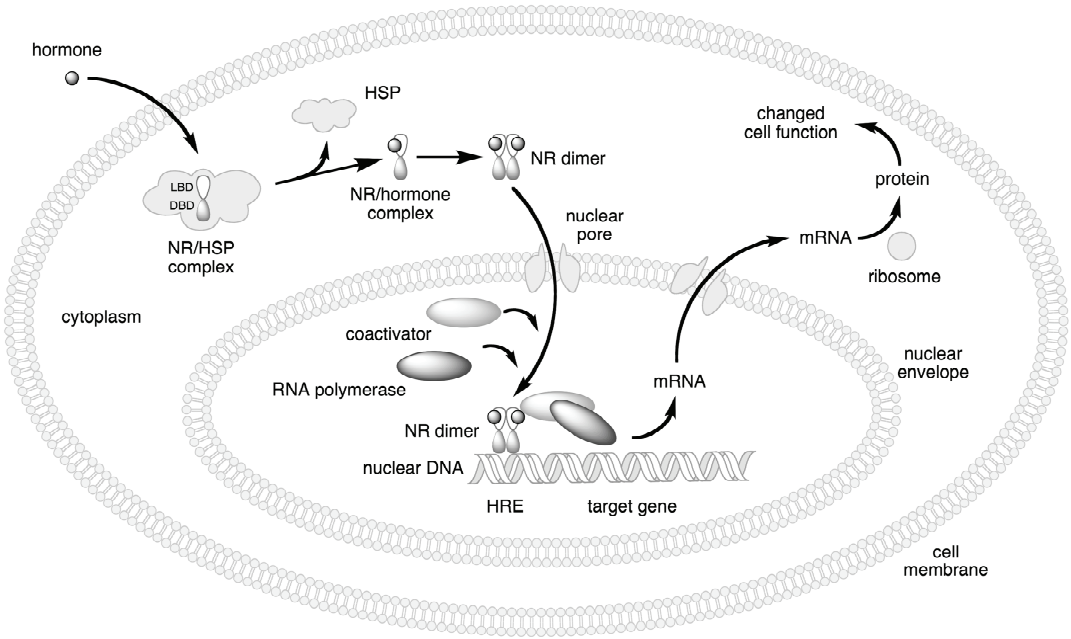


Figure 4. Cellular mechanism corticosteroid receptors. Ligand binding to a nuclear receptor (NR) e.g. GR or MR results in dissociation from the heat shock protein (HSP), dimerization, transloction from the cytoplasm to the nucleus, binding to specific sequences of the DNA called hormone response elements (HRE), recruitment of cofactors and finally transcription of the gene downstream the HRE.

1.1.1. Glucocorticoid receptors

GR are expressed throughout the whole body including the brain. The affinity of GR for cortisol is relatively low therefore cortisol is only bound to GR during stress and at the ultradian peaks when cortisol levels are elevated. Activated GR mediate the metabolic, immunological, cardiovascular and behavioural effects of cortisol in response to stress by mobilizing energy for tissues and cells to cope with a stressor. GR mediated effects are also are aimed at preventing the immunological, inflammatory, cardiovascular and neural responses from overshooting and becoming damaging themselves (Munck et al., 1984). GR is expressed in virtually every cell.

1.1.2. Mineralocorticoid receptors

Mineralocorticoid receptors do not only bind cortisol, they also bind aldosterone with high affinity. Aldosterone operates in the renin-angiotensin system which regulates water/salt homeostasis (see box 1). In epithelial cells of the kidney, colon and sweat glands MR mediate the action of aldosterone on the retention of sodium. In the brain aldosterone selective MR are found in structures involved in maintenance of electrolyte balance such as the medial amygdala, organum vasculosum lamina terminalis (OVLT) and other periventricular brain regions. Epithelial cells express 11β-steroid dehydrogenase type 2 which converts cortisol into cortisone, which has a weak affinity for the receptor, thereby rendering the MR specific for aldosterone (Funder et al., 1988; Edwards et al., 1988).

MR is also expressed in non-epithelial cells in heart, vascular wall and brain and sees in these cells predominantly the naturally occurring glucocorticoids because of their much higher concentration than aldosterone. Thus, the MR is expressed in adipose tissue where it probably mediates cortisol and aldosterone induced adipose tissue development (Zennaro et al., 2009; Caprio et al., 2007) and in heart where the physiological function is still unclear, but the MR seems to contribute to inflammation and fibrosis (Funder, 2009).

In the brain MR is highly expressed in neurons of the limbic brain: hippocampus and amygdala. Due to its high affinity for cortisol, MR in these brain areas is already occupied under basal pulsatile cortisol levels. Therefore it was hypothesized that the MR has a role in regulating these basal cortisol levels; indeed administration of MR antagonists to rats induced elevated corticosterone levels suggesting a role of MR in basal HPA activity (Ratka et al., 1989). Furthermore, also in humans administration of a selective MR antagonist increases basal cortisol levels (Buckley et al., 2007; Wellhoener et al., 2004; Otte et al., 2003a; Otte et al., 2003b; Arvat et al., 2001; Heuser et al., 2000; Deuschle et al., 1998; Young et al., 1998; Born et al., 1997; Dodt et al., 1993b).

Furthermore, MR can bind progesterone. Progesterone is an antagonist for MR and might compete with cortisol for binding under conditions of high circulating progesterone levels e.g. pregnancy, in utero and in the luteal phase of the menstrual cycle (Grossmann et al., 2004; Myles and Funder, 1996).

In addition to the intracellular localization of the receptors membrane bound MRs have been identified (Joels et al., 2008; Karst et al., 2005). These receptors have a lower affinity than the intracellular MRs. The membrane bound MR mediate fast non genomic actions of corticosterone on glutamate transmission in rat brain tissue. In addition, membrane MR decrease post-synaptic hyperpolarization by inhibition of K^+ currents. Probably these fast non-genomic actions mediate the fast negative feedback on pulsatile release of corticosteroids (Atkinson et al., 2008).

Several studies demonstrated an influence of the MR on behaviour. For example, an effect was observed in the Morris water maze when the escape platform was removed after several trainings. The control group remained searching in the quadrant in which previously the platform had been located while the group treated with a MR antagonist searched the water maze for alternative escape routes. It was concluded that MR blockade influenced coping strategy (Oitzl and de Kloet, 1992). Other studies demonstrated that MR blockade inhibits aggressive behaviour in male rats (Haller et al., 1998) and rainbow trout (Schjolden et al., 2009). Furthermore, transgenic mice with increased levels of MR in the forebrain showed decreased anxiety-like behaviour and enhanced memory (Lai et al., 2007). In addition, female transgenic mice, with MR overexpression in the forebrain / hippocampus, show a moderate suppression of the corticosterone response to restraint stress (Rozeboom et al., 2007).

Due to the high affinity of the MR for cortisol, the MR will remain mostly occupied during the hourly pulses of the hormone. Therefore, changes in expression of the MR will be an important level of control of its function. Expression of hippocampal MR is influenced by stress, ageing and corticosteroid treatment (van Eekelen et al., 1992). In addition, estrogens decrease the expression while progesterone increases the expression in rat (Carey et al., 1995; Turner, 1990). It is expected that MR-mediated functions are modulated by ageing, gender and prolonged stress through changes in MR-expression.

1.2. Testing the HPA axis

Several methods are developed to test the different aspects of the HPA axis in humans. Testing the reactivity can be performed by applying a physical or psychosocial stressor or more directly by the administration of ACTH or CRH. Synthetic glucocorticoids can be used to test the negative feedback of the system while inhibition of the HPA axis during basal non stressful situations can be tested by measuring cortisol levels during the day. In this paragraph frequently used and new promising methods for testing the different aspects of the HPA axis are described.

1.2.1. Cortisol

Cortisol can be measured in blood, urine, saliva or hair. In the circulation 80-90% of cortisol is bound to cortisol binding globulin (CBG), 6-15% to albumin and the remaining 4-5% is unbound. The albumin bound and free cortisol fractions are directly available to cells and therefore represent biological active fractions (Rosner, 1990). Reference values for blood cortisol are 0,2 - 0,6 µmol/l in the morning and 0,1 - 0,4 µmol/l in the afternoon. In saliva and urine only the unbound cortisol is present. Usually 24 hours of urine is collected for assessment of cortisol levels, the adult (18

years or older) reference range is 24-108 µg/24 hours. The upper limit of normal for children between 0 and 17 years old is 91 µg/24 hours.

1.2.2. Cortisol Awakening Response

The cortisol awakening response (CAR) is a distinct rise in cortisol levels occurring in response to morning awakening (Wilhelm et al., 2007). Peak values are mostly observed during the first 15-30 minutes. Across several studies in healthy adults, it was reported that salivary cortisol levels increase from about 50% up to over 100% (Clow et al., 2004). In the last decade, the cortisol awakening response (CAR) has been established as a useful marker of HPA axis activity. The function of the CAR is still unclear; a recent review hypothesizes that the cortisol rise after awakening may accompany an activation of prospective memory representations at awakening enabling the individual's orientation about the self in time and space as well as anticipation of demands of the upcoming day (Fries et al., 2009).

The CAR is a relatively easy to measure marker and is assessed by taking saliva samples in the first hour after awakening. A standard procedure has been established with four to five sampling time points, directly after awakening, i.e. 15, 30, 45, and 60 minutes after awakening. The assessment of the CAR by saliva samples has many advantages compared to other methods. The method is non-invasive and can be performed at home; this prevents stress as compared to invasive methods such as needles. Moreover, storage and mailing of samples is possible since cortisol in saliva is quite stable over time. The major disadvantage is the compliance of the participants to follow the strict sampling procedure. With the help of electronic monitoring devices (MEMS track caps) it was demonstrated that the absence of a CAR in a remarkable part of the samples did not reflect absent morning increases but measurement error based on participant's non-compliance with the saliva sampling procedure (Kudielka et al., 2007b; Broderick et al., 2004; Kudielka et al., 2003).

Recent review papers show an overview of findings related to the CAR (Fries et al., 2008; Chida and Steptoe, 2008; Clow et al., 2004). Factors influencing the CAR include gender, depressive symptomatology, PTSD, primary insomnia, chronic fatigue as well as neuroticism and perceived chronic stress (Wessa et al., 2006; Portella et al., 2005; Backhaus et al., 2004; Roberts et al., 2004; Rohleder et al., 2004; Schlotz et al., 2004; Bhagwagar et al., 2003; Pruessner et al., 2003; Wüst et al., 2000a). Moreover, there is a medium-sized, yet distinct heritability of the CAR observed (Bartels et al., 2003; Wüst et al., 2000b).

1.2.3. Dexamethasone suppression test

The dexamethasone suppression test (DST) is used to test the negative feedback of the HPA axis (Carroll et al., 1968). Dexamethasone is a synthetic glucocorticoid that provides negative feedback to suppress the secretion of ACTH. Dexamethasone at low concentrations is relatively unable to pass the blood brain barrier due to transport by P-glycoprotein therefore it is thought that dexamethasone acts predominantly at the pituitary level (de Kloet et al., 1975; de Kloet et al., 1974). Moreover, in MDR-/- mice, which are lacking the P-glycoprotein at the blood brain barrier, dexamethasone was very well capable of entering the brain and binding to both GR and MR (Meijer

et al., 1998). It is proposed that in intact animals and humans, dexamethasone depletes the brain of its endogenous ligand corticosterone / cortisol, by inhibition of ACTH production (Karssen et al., 2005; Karssen et al., 2001).

1.2.4. CRH Stimulation Test

The CRH stimulation test is used to asses the responsivity of the pituitary to ACTH. CRH is given intravenously as a bolus injection and directly after the injection blood samples are taken every 5-10 minutes for ACTH and cortisol measurements. In healthy individuals the increase in ACTH is observed 5-15 min after the CRH administration and the increase in cortisol is observed after 30-60 minutes (Gold et al., 1988). In depressed individuals this CRH-induced cortisol response is blunted (Holsboer, 1986).

1.2.5. Dexamethasone-CRH Test

The dexamethasone-CRH (Dex-CRH) test was developed as a refined DST procedure. The test combines the dexamethasone suppression and the CRH stimulation tests. Dexamethasone is administered orally at 11pm, then on the next day CRH is injected and blood samples are taken every 15 minutes. The advantage of this procedure is that at the moment of CRH administration the HPA axis is downregulated due to the feedback inhibition by dexamethasone (Heuser et al., 1994; Bardeleben and Holsboer, 1989). There is a strong correlation between the cortisol responses in the DST and the Dex-CRH test, however the sensitivity of the Dex-CRH test is better (Watson et al., 2006). For instance during severe depression the escape of cortisol from dexamethasone suppression is enhanced by CRH; this effect could be a result of increased vasopressin (AVP) expression in the PVN. Hence depressed patients are showing an exaggerated Dex-CRH response (Watson et al., 2006).

1.2.6. ACTH stimulation test

The ACTH stimulation test (also called the cosyntropin test or tetracosactide test) is used to assess the functioning of the adrenal glands. A small amount of synthetic ACTH (cosyntropin) is injected which stimulates the adrenals to release cortisol and sometimes aldosterone.

1.2.7. Trier Social Stress Test

The Trier Social Stress test (TSST) is a psychological procedure that induces stress under laboratory conditions. The TSST is a motivated performance task consisting of a brief preparation period (3 minutes) followed by a test period in which the subject has to deliver a free speech (5 minutes) and perform mental arithmetic (5 minutes) in front of an audience. Outcome variables range from subjective-verbal stress reports to objective behavioral and biological stress responses including parameters of the HPA axis like ACTH and cortisol, the cardiovascular, immunological, and blood coagulation system. The TSST can be applied in younger and older adults, in children as well as in clinical populations reviewed by (Kudielka et al., 2009). The TSST combines elements of uncontrollability and high levels of social-evaluative threat. Stress tasks containing the two

components ‘uncontrollability’ and ‘social-evaluative threat’ are associated with the largest stress responses and the longest recovery times (Dickerson and Kemeny, 2004).

1.3. Psychopathology and the HPA axis

Stress had been implicated in the etiology of several psychiatric disorders and disregulation of HPA axis regulation has been reported frequently in patients with psychiatric disorders. The “three hit model” proposes that genetic variation (hit 1) in interaction with early life experiences (hit 2) determines vulnerability or resilience to develop a psychiatric disorder after a stressful event (hit 3) later in life (Figure 1). Three stress-related psychiatric disorders are discussed in more detail in this paragraph.

1.3.1. Major depressive disorder

Major depressive disorder (also known as clinical depression, major depression, unipolar depression, or unipolar disorder) is a mental disorder characterized by an all-encompassing low mood accompanied by low self-esteem, and loss of interest or pleasure in normally enjoyable activities. It is a disabling condition which adversely affects a person's family, work or school life, sleeping and eating habits and general health.

The term "major depressive disorder (MDD)" was selected by the American Psychiatric Association to designate this symptom cluster as a mood disorder in the 1980 version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III) classification, and has become widely used since. The general term depression is often used to describe the disorder, but as it is also used to describe a more temporarily depressed state of mind, more precise terminology is preferred for the disorder in clinical and research use. The most widely used criteria for diagnosing depressive conditions are found in the American Psychiatric Association's revised fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and the World Health Organization's International Statistical Classification of Diseases and Related Health Problems (ICD-10), which uses the name recurrent depressive disorder.

The lifetime prevalence of MDD is at least 10% with the risk in women twice as high as in men. Hereditability is determined based on twin studies and is 40 to 50%. Environmental risk factors include childhood abuse and neglect and life stress (Kendler et al., 2004; Kendler et al., 2002)

In 1976 Carroll et al tested different aspects of the HPA axis in patients with MDD. Patients frequently have high cortisol levels but the diurnal rhythm is usually maintained and they usually do not develop the physical signs of Cushing’s syndrome. However, depression is a common symptom of Cushing’s syndrome. The feedback mechanism of the HPA axis was tested in depressed patients with the dexamethasone suppression test using 1 mg dexamethasone. Forty percent of the patients showed an abnormal HPA axis feedback inhibition with an early escape from dexamethasone suppression. However, not all studies show such a high percentage non-suppressors. It is quite possible that specific subgroups of depressed patients, such as patients with comorbid psychotic features account for the non-suppression.

1.3.2. PTSD

Posttraumatic stress disorder (PTSD) is an anxiety disorder; it is a severe and ongoing emotional reaction that can develop after exposure to an extreme stressor. This stressor may involve a threat to the patient's or someone else's life, serious physical injury, an unwanted sexual act, a threat to physical or psychological integrity or overwhelming psychological defenses. In some cases it can also result from profound psychological and emotional trauma, without any actual physical harm. Symptoms include re-experience such as flashbacks and nightmares, avoidance of stimuli associated with the trauma, increased arousal such as difficulty falling or staying asleep, anger and hyper vigilance. Per definition, the symptoms last more than six months and cause significant impairment in social, occupational, or other important areas of functioning e.g. problems with work and relationships.

Many studies investigated the reactivity of the HPA axis in patients with PTSD (reviewed by (de Kloet et al., 2006)). Baseline studies in adult PTSD patients report high CRH in cerebrospinal fluid, while diurnal plasma cortisol levels on average are decreased. Single point plasma and 24 hours urine cortisol levels reveal mixed results. Lower plasma and 24 h cortisol levels have been reported in some but not in other studies (Yehuda, 2002). In most studies PTSD patients show an enhanced suppression of cortisol after 0.5 or 1 mg dexamethasone.

1.3.3. Burnout

Burnout is a psychological term for the experience of long-term exhaustion and diminished interest. Service and people oriented professionals such as teachers, health practitioners, care givers, fire fighters and police men seem more prone to burnout than others (Melamed et al., 2006; Maslach et al., 2001).

Burnout is not a recognized disorder in the DSM-IV. The most extensively studied measurement of burnout in the literature is the Maslach Burnout Inventory. Maslach and her colleague Jackson first identified the construct "burnout" in the 1970s, and developed a measure that weighs the effects of emotional exhaustion and reduced sense of personal accomplishment. This indicator has become the standard tool for measuring burnout in research on the syndrome. People who experience all three symptoms have the greatest degrees of burnout, although emotional exhaustion is the hallmark of burnout.

There are several studies testing HPA axis functioning in burnout. However the results are inconsistent, some studies report no association between basal cortisol levels and burnout (Langelaan et al., 2006; Mommersteeg et al., 2006) while others find higher (Grossi et al., 2005; De et al., 2003) or lower (Sonnenschein et al., 2007; Pruessner et al., 1999) levels. Also studies on HPA axis feedback tested with 0.5 mg dexamethasone gave contradictory results.

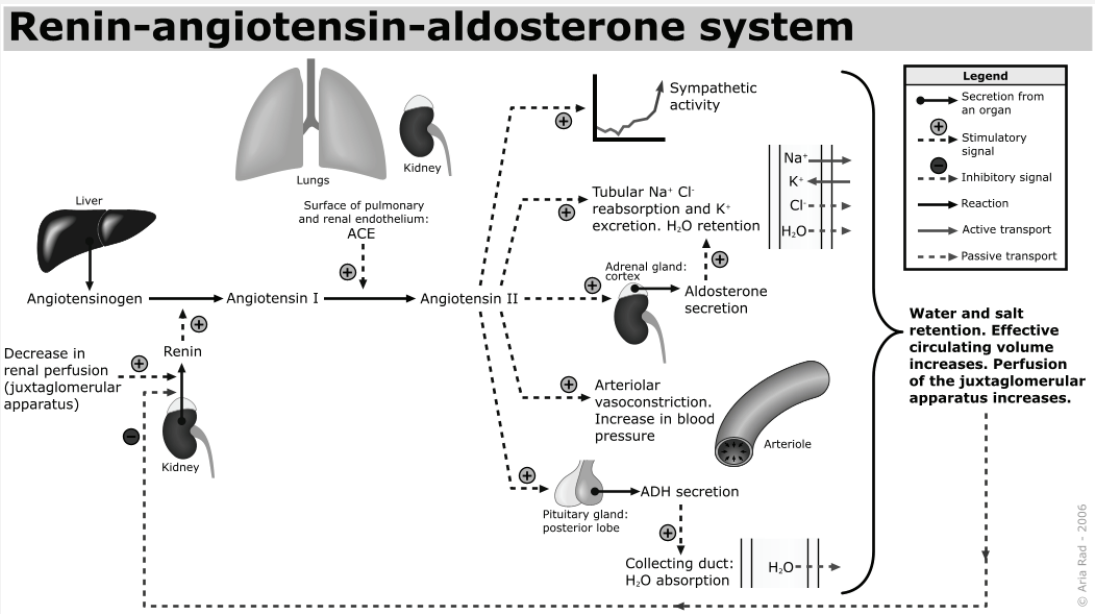
Box 1 The MR and the Renin Angiotensin System

Text and figure adapted from www.wikipedia.org

The renin-angiotensin-aldosteron system (RAS) is a hormone system that regulates blood pressure and water (fluid) balance.

When blood volume is low, the kidneys secrete renin, in turn renin stimulates the production of angiotensin that causes blood vessels to constrict, resulting in increased blood pressure. In addition to the effect on the blood vessels, angiotensin stimulates the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone promotes Na+ and water retention, and lowers plasma K+ which also increases blood pressure concentration by different mechanisms First of all, aldosterone acts on the MR within the principal cells of the distal tubule and the collecting duct of the kidney nephron, this up regulates and activates the basolateral Na+/K+ pumps, stimulating ATP hydrolysis leading to phosphorylation of the pump and a conformational change in the pump exposes the Na+ ions to the outside. The phosphorylated form of the pump has a low affinity for Na+ ions, hence reabsorbing sodium (Na+) ions and water into the blood, and secreting potassium (K+) ions into the urine. Secondly, aldosterone up regulates epithelial sodium channel (ENaC) increasing apical membrane permeability for Na+, Cl- is reabsorbed in conjunction with sodium cations to maintain the system's electrochemical balance. Furthermore, aldosterone stimulates uptake of K+ into cells and H+ secretion by intercalated cells in the collecting duct, regulating plasma bicarbonate ($HCO3^-$) levels and its acid/base balance. In addition aldosterone stimulates Na+ and water reabsorption from the gut salivary and sweat glands in exchange for K+ and aldosterone may act on the central nervous system via the posterior pituitary gland to release vasopressin (AVP), which serves to conserve water by direct actions on renal tubular reabsorption

If the RAS is too active, blood pressure will be too high. There are many drugs that interrupt different steps in this system to lower blood pressure. These drugs are one of the main ways to control high blood pressure (hypertension), heart failure, kidney failure, and harmful effects of diabetes.



1.1. Testing of the renin angiotensin system

The RAS can be tested under basal conditions and there are several methods for testing activation of the system. The most common used measures and tests are described here.

1.1.1. Blood pressure

Blood pressure (BP) is a force exerted by circulating blood on the walls of blood vessels, and is one of the principal vital signs. During each heartbeat, BP varies between a maximum (systolic) and a minimum (diastolic) pressure. The mean BP, due to pumping by the heart and resistance in blood vessels, decreases as the circulating blood moves away from the heart through arteries. It has its greatest decrease in the small arteries and arterioles, and continues to decrease as the blood moves through the capillaries and back to the heart through veins. Gravity, valves in veins, and pumping from contraction of skeletal muscles, are some other influences on BP at various places in the body.

The term blood pressure initially refers to the pressure measured at a person's upper arm. It is measured on the inside of an elbow at the brachial artery, of which the measurements can be conclusive, which is the upper arm's major blood vessel that carries blood away from the heart.

While average values for arterial pressure could be computed for any given population, there is often a large variation from person to person; arterial pressure also varies in individuals from moment to moment. Additionally, the average of any given population may have a questionable correlation with its general health, thus the relevance of such average values is equally questionable. However, in a study of 100 subjects with no known history of hypertension, an average blood pressure of 112/64 mmHg was found (Pesola et al. 2001) which is in the normal range.

Various factors influence a person's average BP and variations. Factors such as age and gender (Reckelhoff et al. 2001) influence average values. In children, the normal ranges are lower than for adults and depend on height (http://www.nhlbi.nih.gov/guidelines). As adults age, systolic pressure tends to rise and diastolic tends to fall (Pickering et al. 2005). In the elderly, BP tends to be above the normal adult range (Pickering et al. 2005) largely because of reduced flexibility of the arteries. Also, an individual's BP varies with exercise, emotional reactions, sleep, digestion and time of day.

Classification of blood pressure for adults

Category	**systolic, mmHg**	**diastolic, mmHg**
Hypotension	< 90	< 60
Normal	**90 – 120**	**60 – 80**
Prehypertension	121 – 139	or 81 – 89
Stage 1 Hypertension	140 – 159	or 90 – 99
Stage 2 Hypertension	≥ 160	or ≥ 100

All levels of arterial pressure put mechanical stress on the arterial walls. Higher pressures increase heart workload and progression of unhealthy tissue growth (atheroma) that develops within the walls of arteries. The higher the pressure, the more stress that is present and the more atheroma tend to progress and the heart muscle tends to thicken, enlarge and become weaker over time. Persistent hypertension is one of the risk factors for strokes, heart attacks,

heart failure and arterial aneurysms, and is the leading cause of chronic renal failure. Even moderate elevation of arterial pressure leads to shortened life expectancy. At severely high pressures, mean arterial pressures 50% or more above average, a person can expect to live no more than a few years unless appropriately treated (Guyton & Hall)

In the past, most attention was paid to diastolic pressure; but nowadays it is recognised that both high systolic pressure and high pulse pressure (the numerical difference between systolic and diastolic pressures) are also risk factors. In some cases, it appears that a decrease in excessive diastolic pressure can actually increase risk, due probably to the increased difference between systolic and diastolic pressures

1.1.2. 24 Hours sodium excretion

Urine is collected for 24 hours and sodium concentrations are measured. Reference values are: 40-100 mmol/24h. Lower than normal urine sodium levels may indicate: Aldosteronism, Congestive heart failure, Diarrhea and fluid loss, Kidney failure. Greater than normal urine sodium levels may be caused by too much salt in the diet or certain medications. High concentrations sodium are associated with higher blood pressure.

1.1.3. Plasma aldosterone

Aldosterone is measured in plasma reference values are: lying down: 2 to 16 ng/dL and upright: 5 to 41 ng/dL. Lower than normal levels of aldosterone may indicate: Addison's disease (rare), Congenital adrenal hyperplasia, Hyporeninemic hypoaldosteronism or a very high-sodium diet. Higher than normal levels of aldosterone may indicate: Bartter syndrome (extremely rare), Primary hyperaldosteronism (rare), Secondary hyperaldosteronism from heart or kidney disease or a very low-sodium diet

1.1.4. Renin activity

The enzyme activity in plasma is measured and normal values range from 1.9 to 3.7 ng/mL/hour. Normal value ranges may vary slightly among different laboratories. Higher than normal levels may indicate: Addison's disease, Cirrhosis, Dehydration, Hemorrhage (bleeding), High blood pressure, Hypokalemia, Malignant hypertension, Nephrotic syndrome, Renin-producing renal tumors, Renovascular hypertension. Lower than normal levels may indicate: ADH therapy, Sodium-retaining steroid therapy, Sodium-sensitive high blood pressure.

1.1.5. Acute salt loading and salt depletion

Acute salt-loading (constant rate intravenous infusion of 2 L of 0.9% NaCl carried out over 4 hours) and salt-depletion protocol (sodium restriction 50 mmol plus three doses of 37.5 mg of furosemide) is used to evaluate the distribution of blood pressure sensitivity to salt (strazullo 2000). If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period was greater than the median (10 mmHg), the patient was classified as “salt-sensitive” otherwise he or she was considered “salt-resistant. Post-load plasma aldosterone and renin activity were measured 4 h after the beginning of the salt-load.

1.1.6. Low Na+ and high K+ or high Na+ and low K+ diet

Salt sensitivity is tested with a crossover study with low Na+ and high K+ / high Na+ and low K+ for 1 week. Individuals receive both a low Na+ (less than 20 mmol NaCl/day) and high K+ (more than 140 mmol Kcl/day; low Na+–high K+ diet) or high Na+ (more than 250

mmol NaCl/day) and low K+ (less than 50 mmol Kcl/day; high Na+–low K+ diet) for 1 week. Controlled Na+/K+ diet periods were separated by a 7-day washout period. On the ad libitum Na+ and K+ diet at baseline and on day 7 of each controlled Na+/K+ diet period, blood was sampled at 0900 in the fasting state after 1 hour of rest in the sitting position for plasma immunoreactive active and total renin and plasma aldosterone and ANP determinations. Urine was collected in 2 12-hour periods from 0800 to 2000 and from 2000 to 0800 and plasma active renin, total renin, atrial natriuretic peptide (ANP) and aldosterone were measured.

2. Genetic variation

2.1. Types of genetic variation

Human genetic variation underlies the total amount of genetic characteristics observed within the human species. Genetic differences are observed between humans at both the individual and the population level. There are multiple variants of one gene in the human population and these variants of the gene are called alleles.

Several events can lead to genetic variation including random mutations of one nucleotide in the DNA and the exchange of genes during meiosis. There are two reasons why genetic variation exists between populations. First, there is natural selection in which a specific allele may confer an advantage to individuals in a specific environment and this can lead to an advantage in reproduction of this individual. The second reason is the neutrality of most mutations. Most mutations do not have a direct obvious effect on the body and will therefore be passed on without any selection or prevalence. However, it cannot be ruled out that under certain extreme or specific conditions changes in function will appear.

Genetic variation among individual humans occurs on many different scales, ranging from complete duplications of a chromosome to single nucleotide changes.

2.1.1. Single nucleotide polymorphisms

A single-nucleotide polymorphism (SNP, pronounced *snip*) is the most common DNA variation. It is a single nucleotide difference in the genome between members of a species or between the two chromosomes of an individual. The nucleotide can either be substituted, deleted or inserted. The nucleotide diversity between humans is about 0.1% resulting in approximately 3 million nucleotide differences since the human genome has around 3 billion nucleotides.

Most of the SNPs are neutral but some are functional and influence phenotypic differences between humans. SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same amino acid sequence is termed *synonymous;* if a different

amino acid sequence is produced they are *nonsynonymous*. A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, mRNA binding or stability of the mRNA.

2.2. Human Mineralocorticoid Receptor gene

2.2.1. Gene structure, splice and translational variants

The gene coding for the human MR (hMR) is located on chromosome 4 at position q31.1. The gene consists of 10 exons (Figure 6). Exons 2 to 9 form the coding region with exon 3 and 4 coding for the DNA binding domain, exon 5, 6, 7, 8 and the first part of exon 9 coding for the ligand binding domain. There are two known 5'-untranslated exons named 1α and 1β, generating the mRNA isoforms hMRα and hMRβ. Two different promoter regions named P1 and P2 are located upstream of exon 1α and 1β, respectively. The complete human MR protein is composed of 983 amino acids and has a molecular mass of 107 kDa.

Several mRNA splice variants have been described. The variant hMRΔ5,6 lacks exons 5 and 6 resulting in a protein of 75 kDa lacking the entire hinge region and the ligand binding domain of the receptor (Zennaro et al., 2001). The use of an alternative splice site after exon 3 results in a variant containing four extra amino acids in the first zinc finger of the DNA binding domain (Bloem et al., 1995).

In addition to the mRNA splice variants, hMR protein diversity is also created by the translation from two translation start sites resulting in the 107kDa MR-A and the 15 amino acid smaller 105.4kDa MR-B (Pascual-Le Tallec et al., 2004) (Figure 6).

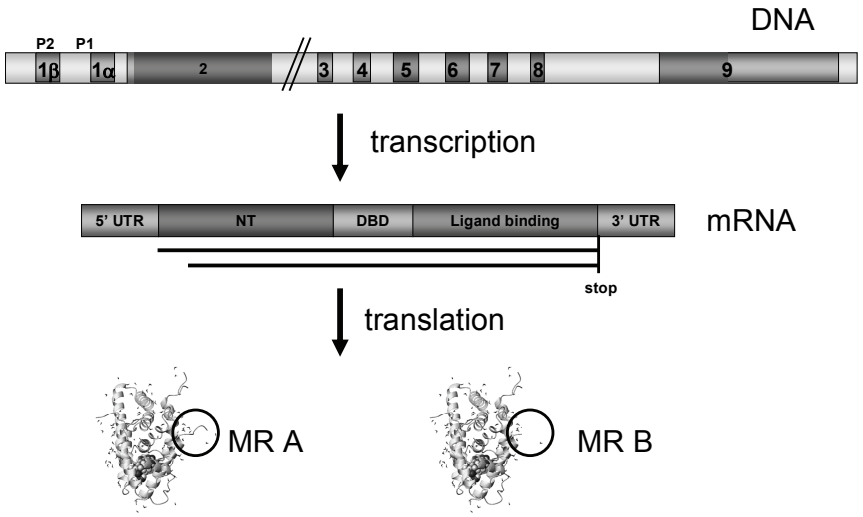


Figure 6.
Schematic representation of the human MR gene, MR mRNA and the two translational protein variants of the MR, named MR-A and MR-B. In the MR DNA the intronic regions are indicated in light gray, the dark gray areas indicate the exonic regions and correspond to the dark gray areas in the mRNA. In the mRNA UTR= untranslated region, NT= N-terminal domain and DBD= region coding for the DNA binding domain. The two black lines below the mRNA indicate the two possible translated areas of the mRNA resulting in the 107KDa MR-A and the 105.4kDA MR-B

2.2.2. Genetic variation

Genetic variation in the MR is reported in several studies (Figure 7). Associations with low frequent (<1% in the population) MR mutations have been found to be associated with rare genetic disorders while the more frequent (>1% in the population) single nucleotide polymorphisms have been found to be associated with common variation in healthy individuals.

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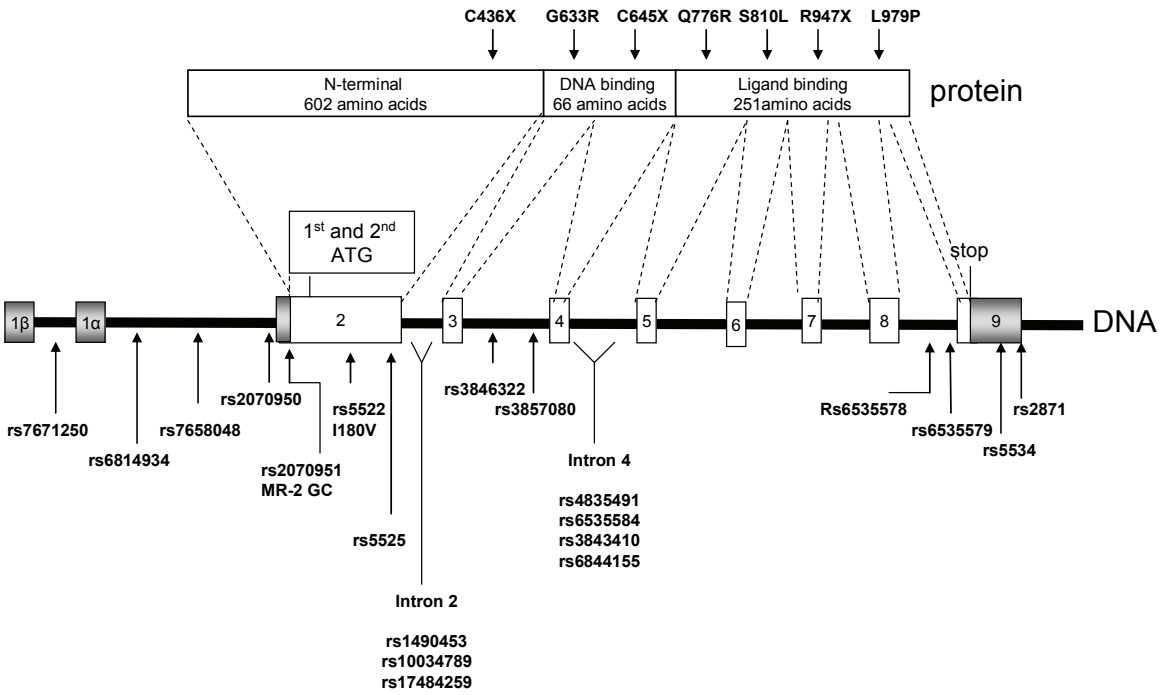


Figure 7. structure of the MR gene, intronic DNA regions are displayed as the black line in the middle, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA, while the protein structure and mutations are displayed above.

The rare MR mutations called R947X, C436X, c.1132-1133insT, c.315del8bp, C645X, G633R, Q776R and L979P have been associated with pseudohypoaldosteronism type I (PHA1). Pseudohypoaldosteronism type 1 is a rare condition characterized by electrolyte disorders caused by the kidney's inability to respond to mineralocorticoids. There are two forms: an autosomal recessive form which tends to be more severe than the autosomal dominant form. The recessive form tends to persist into adulthood whereas the dominant form is milder and symptoms tend to improve with age. *In vitro* studies showed that the mutations involved in PHA1 give a loss of the MR transactivation capacity (Fernandes-Rosa et al., 2006; Riepe et al., 2004; Nystrom et al., 2004; Riepe et al., 2003).

The S810L (rs414511344) mutation has been associated with hypertension in young women which increases during pregnancy resulting in the medical condition pre-eclampsia (Geller et al., 2000). However, other studies did not find an association with this mutation and hypertension or severe

pre-eclampsia (Martinez et al., 2009; Tempfer et al., 2004; Sugiyama et al., 2001). In vitro studies demonstrated that this mutation creates a gain of transactivational capacity of the MR (Geller et al., 2000).

Two SNPs in the MR were tested for *in vitro* functionality. One of these SNPs is called MRI180V (rs5522) and is located in exon 2. This SNP results in an isoleucine to valine amino acid change in the N-terminal domain of the protein. One study found that the SNP decreased the transactivation capacity of the MR *in vitro* using cortisol as a ligand while in this study no differences were found with aldosterone (DeRijk et al., 2006). However, another study demonstrated significant differences in transactivation using aldosterone (Arai et al., 2003). In that study the maximal transactivation of MR180V is observed at a concentration of 10^{-10}M aldosterone and is 20% lower compared to the maximal transactivation of MRI180 which is observed at 10^{-8}M aldosterone. MR180V is associated with enhanced cortisol, and autonomic responses to an acute psychosocial stressor measured with the TSST (DeRijk et al., 2006), more feelings of depression (Kuningas et al., 2007) and protection against hypertension (Martinez et al., 2009). Other studies did not find an association with blood pressure (Tobin et al., 2008). Also the enhanced cortisol response to the TSST could not be confirmed in a smaller study, however in this study there was an association between MR180V and higher ACTH responses and anxiety during the second TSST performed in this group (Ising et al., 2008).

The second SNP studied in the MR was MR-2G/C (rs2070951), this SNP has hardly been tested; therefore in this thesis the functionality of this SNP will be further demonstrated. The SNP is located 2 nucleotides before the first translational startsite of the MR in the Kozak consensus sequence and it is hypothesized that this SNP changes the translation and/or the MR-A / MR-B balance. One *in vitro* study demonstrated a decrease in transactivational capacity using aldosterone with the C variant compared to the G variant (Arai et al., 2003). Furthermore, the C variant of MR-2G/C is associated with lower basal cortisol levels (Kuningas et al., 2007). No associations were found between this SNP and hypertension (Martinez et al., 2009) or blood pressure (Tobin et al., 2008).

Two studies tested multiple SNPs covering the whole MR for an association with blood pressure. In one study only the in vitro tested MRI180V was associated with hypertension (Martinez et al., 2009). In the other study four SNPs located in intron 4 (rs6844155, rs3843410, rs6565584 and 4835491) were associated with clinical diastolic blood pressure (DBP), a SNP in intron 3 and a SNP in intron 2 (rs3846322 and rs17484259) were associated with clinical SBP, one SNP in intron 3 (rs3857080) was associated with mean night time DBP and SBP and one SNP in intron 2 was associated with mean night DBP (Tobin et al., 2008). These studies indicate involvement of the MR in blood pressure, which was already known based on other studies. The mechanism of action of these SNPs is still unclear since they were not tested *in vitro* for functionality. In addition, the associations need replication in a second cohort because these findings are only exploratory due to the large number of analysis performed.

Furthermore a genome wide association study revealed an association between SNP rs1490453 located in intron 2 and fibrinogen levels (Zemunik et al., 2009). Again this finding is only explorative and needs replication in a second cohort.

2.3. The glucocorticoid receptor gene

2.3.1. Gene structure, splice and translational variants

The gene coding for the human GR (hGR) is located on chromosome 5 at position q31.32. The gene consists of the translated exons 2 to 9 and at least seven 5'-untranslated exons 1 named 1A to 1F. In accordance with the MR exons 3 and 4 are coding the DNA binding domain, exons 5, 6, 7, 8 and a part of exon 9 are coding for the ligand binding domain. Several splice variants have been described. Exon 9 consists of two parts, 9α and 9β and this can result in a mRNA containing 9α mRNA, 9β or both 9α and 9β (Oakley et al., 1996).

2.3.2. Genetic variation

Many studies investigated the role of SNPs in the GR in HPA axis functioning and stress-related disorders.

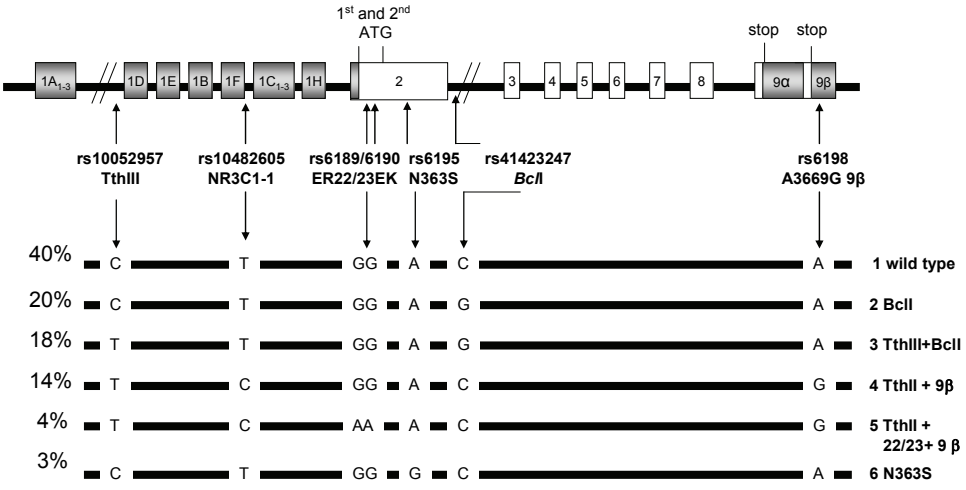


Figure 8. Structure and haplotypes of the hGR gene. Intronic DNA regions are displayed as the black line in the midle, exonic regions are displayed as the boxes on the line. SNPs and haplotypes are indicated below the DNA

In the promoter region of the GR gene, between exon 1A and 1D is a SNP changing a TthIII restriction site (rs100529570). This SNP has not been tested for functionality *in vitro* but is associated with higher basal cortisol levels in men (Rosmond et al., 2000). A more recently described SNP in the promoter region is NR3C1-1 (rs10482605). This SNP is located 30bp downstream of exon 1F. *In vitro* tests demonstrated that the minor allele (C) results in a lower

transcriptional activity and this C allele is associated with higher post dexamethasone (0.25mg) plasma ACTH and cortisol in men, but with lower levels in women (Kumsta et al., 2008; Kumsta et al., 2007).

In exon 2 there are two SNPs that always occur together, they are located in codon 22 and 23 and the sequence change in codon 23 results in an Arginine (K) to lysine (R) amino acid change. These SNPs are named EK22/23ER (rs6189 and rs6190) and *in vitro* they change the transactivation capacity but not the repression capacity of the GR. They are associated with higher post-dexamethasone cortisol levels (corticosteroid resistance), low insulin, glucose and CRP levels, beneficial body composition, more muscle strength, better survival, less dementia and white matter lesions and higher risk for major depression (Van Rossum et al., 2006; Van Rossum et al., 2004a; Van Rossum et al., 2004b; Van Rossum et al., 2002).

Further upstream in exon 2 the Asparagine to Serine changing SNP N363S is located. This SNP changes the transactivation capacity in both transfection experiments (*in vitro*) and in lymphocytes of carriers of the SNP (*ex vivo*). N363S is associated with lower post dexamethasone morning cortisol, higher cortisol levels following the TSST in males, in contrast to females who show lower cortisol levels following the TSST. Some studies found associations with body mass index, but others did not.

In intron B, 647 nucleotides downstream of exon two is the BclI restriction site changing SNP, BclI (rs41423247), located. This SNP is associated with increased corticosteroid sensitivity using the skin bleaching test with the synthetic glycocorticoid beclomethasone (Panarelli et al., 1998), while there was no effect on dexamethasone suppression of LPS-stimulated interleukin-6 production in whole blood cells. Furthermore, this SNP is associated with increased morning cortisol suppression after a low dose dexamethasone (0.25mg), heterozygotes of the SNP had higher cortisol and ACTH responses following the TSST and higher cortisol responses after administration of ACTH compared to both homozygote groups while homozygotes of the minor allele had an increased risk of developing major depression compared to heterozygotes and homozygotes of the common allele (Van Rossum et al., 2006).

SNP 9β (rs6189) is located in exon 9β in the untranslated region of the mRNA. The SNP changes the stability of 9β mRNA resulting in an increased 9β protein expression (DeRijk et al., 2001). The SNP is associated with higher cortisol and ACTH levels in males after administration of dexamethasone (0.25mg) and during the TSST, but in females the SNP was associated with lower levels (Kumsta et al., 2007).

Most studies report the effects of single SNPs. However, some SNPs are linked to each other completely or to a certain extent. The promoter SNP NR3C1-1 is always observed together with the 9β SNP. This is reflected in the associations found with the single SNPs since similar associations were found for NR3C1-1 and 9β. The minor allele of EK22/23EK is always observed with the minor alleles of Tth111I, NR3C1-1 and 9β. However, the minor alleles of Tth111I, NR3C1-1 and 9β are also observed without EK22/23ER. In addition to these frequently tested SNPs, additional

GR SNPs are reported on the NCBI website. These SNP might be linked to the reported GR SNPs and might be functional as well.

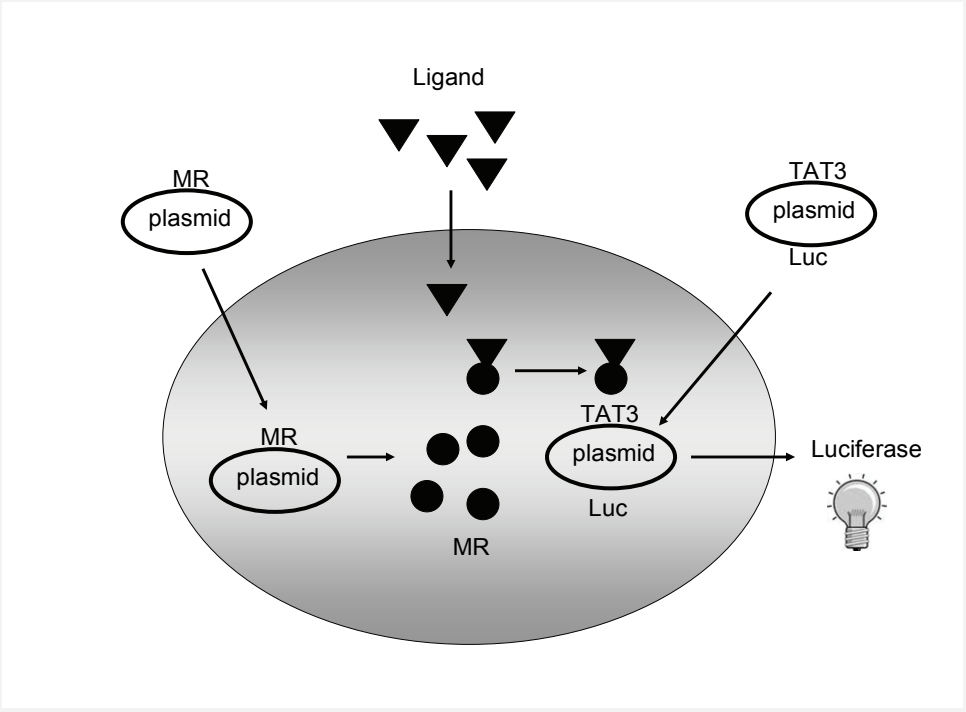
3. Scope and outline of this thesis

The project was designed to test if genetic variation in the MR gene is a risk factor for developing major depression. To establish this, several steps were taken.

First the MR gene was screened for genetic variation in the form of Single Nuclotide Polymorphisms (SNPs) (**chapter 2**). Based on location, two SNPs were selected and tested for *in vitro* functionality because *in vitro* functional SNPs are expected to have *in vivo* effects. The *in vitro* functionality of MR gene SNPs was tested at different levels; on protein and mRNA expression (**chapter 5**), on transactivation capacity (assay explained in **Box 2** and **chapter 3, 4 and 5**) and on ligand binding (**chapter 4 and 5**).

Box 2 Transactivation assay MR

In the transactivation assay cells with no endogenous MR are transfected with a plasmid containing the MR gene. MR will be formed in the cells and a ligand is added to activate the formed MR. Activated MR will activate the reporter plasmid that was co-transfected. The reporter plasmid contains a MR responsive promoter (TAT3) and luciferase reporter gene (Luc) ativated MR will activate this promoter and luciferase is formed. Luciferase is measured and this luciferase expression is a measure of transactivational capacity of the MR.



Second, the two selected functional SNPs were subsequently tested for their influence on stress responsiveness and electrolyte regulation. In a cohort with healthy individuals (n=218) the two SNPs were tested for an association with the cortisol awakening response (CAR) with and without administration of a low dose of the synthetic glucocorticoid dexamethasone (**chapter 3**).

Furthermore, in a group of healthy school teachers (n=157) the SNPs were tested for associations with chronic stress (**chapter 5**) and the response to psychosocial stress measured with the TSST (**chapter 5**). In addition, one SNP was tested for its influence on blood pressure and salt regulation since the MR is not only an important receptor in de HPA axis it is also involved in the Renin-Angiotensin Aldosterone system, regulating salt/water homeostasis (**chapter 4**).

Finally, the functional MR SNPs modulating HPA axis regulation were tested for association with mood, anxiety and somatoform disorders. This was performed in an extensively phenotyped group of controls and patients in which several psychological and biological markers are also available (**chapter 6**).

2

Identification of genetic variation in the Mineralocorticoid Receptor

Nienke van Leeuwen, Andrew Bradley, Birgitta de Deugd, Heleen van den Heuvel, Frans G. Zitman, E. Ron de Kloet & Roel H. de Rijk

Introduction

Genetic variation in the mineralocorticoid receptor (MR) is reported in several studies. Associations with low frequent (<1% in the population) MR mutations have been found to be associated with rare genetic disorders while the more frequent (>1% in the population) single nucleotide polymorphisms (SNPs) have been found to be associated with common variation in healthy individuals, this is described in more detail in chapter 1, paragraph 2.2 of this thesis.
In this study we tried to identify SNPs in the MR, therefore the exonic regions of the MR were screened in fifty individuals from the Leiden population. In addition we performed a computer screening on the genetic variation in the MR to predict possible functionality, we checked if the genetic variation was linked and if haplotypes exist in the MR.

Materials and Methods

Screening exonic regions MR

Fifty anonymous EDTA blood samples were obtained from the general physician laboratory in Leiden, the Netherlands. DNA was isolated using a standard NaCl salting out procedure (Miller et al. 1988). Eighteen primer pairs were developed for PCR reactions covering all exonic regions using Primer3 software (Steve Rozen and Helen J. Skaletsky, 2000; primer sequences on request). All PCR reactions were performed with 50ng DNA, 8.0pM of each primer and AmpliTaq Gold (Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands), according to the manufacturer's protocol. Sequencing of the PCR product was done with BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands) on an ABI Prism 3100-Avant Genetic Analyzer, according to the manufacturer's instructions, while sequences were analyzed using Chromas Lite (Technelysium Pty Ltd) and Vector NTI (Invitrogen).

Computer screening DNA sequences

The program TFSEARCH (http://www.rwcp.or.jp/papia/ Heinemeyer 1998) was used to identify transcription factor binding sites, UTR-scan was used to identify regulatory regions in the untranslated regions and targetscan (http://www.targetscan.org) was used to identify microRNA target regions.
DNA sequences with and without the SNPs were subjected to the computer programs to reveal a possible effect of the SNP on transcription factor binding sites, regulatory regions and microRNA target regions.

Results

MR SNP identification

The exonic regions of the human MR gene were screened for genetic variation by direct sequencing fifty individuals from the Dutch population. There were no drop outs, all exonic regions were measured in all individuals. Seven SNPs were found in the exonic regions, rs2070951 (Minor allele frequency (MAF) 47%), rs5522 (MAF 11%), rs5523 (MAF 1%), rs5525 (MAF 11%), rs5528 (MAF 1%), rs5534 (MAF 44%) and rs2871 (MAF 22%). No new SNPs were identified, all SNPs were described in the NCBI database (December 2008).

Computer screenings showed that rs2070951 is located in the first Kozak sequence of the gene; this region determines translation of the protein. Other SNPs were not located at predicted transcription binding sites, regulatory regions or microRNA target regions. However rs2871 appeared to be located at a region that is prone to loop formation. This was identified by the primer design software, during development of primers the primer design software indicated possible loop formation of the sequence.

MR genotypes and haplotypes

Linkage Disequilibrium (LD) and haplotype analyses revealed two haplotype blocks. One, containing the three high frequent SNPs in exon 2 rs2070951, rs5522 and rs5525. The other, contains the two high frequent SNPs in exon 9 rs5534 and rs2871 (Fig 1 and 2).

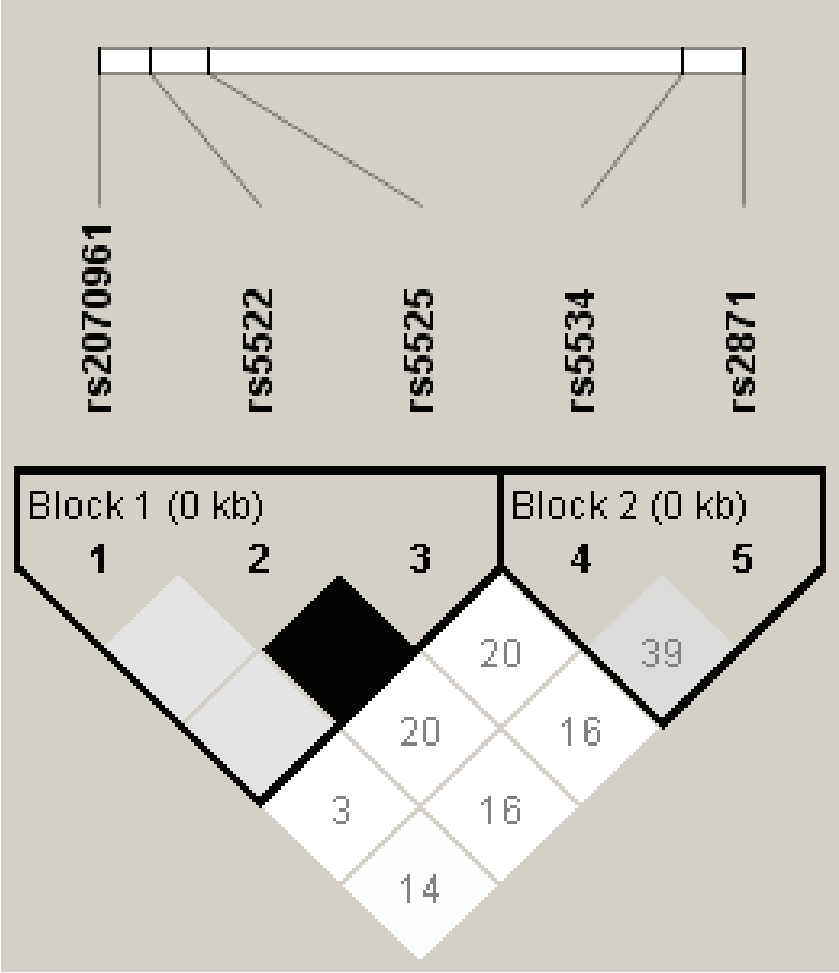


Figure 1. LD between MR SNPs in r^2. Black squares indicate $r^2 = 1$ and shades of grey indicate $r^2 < 1$

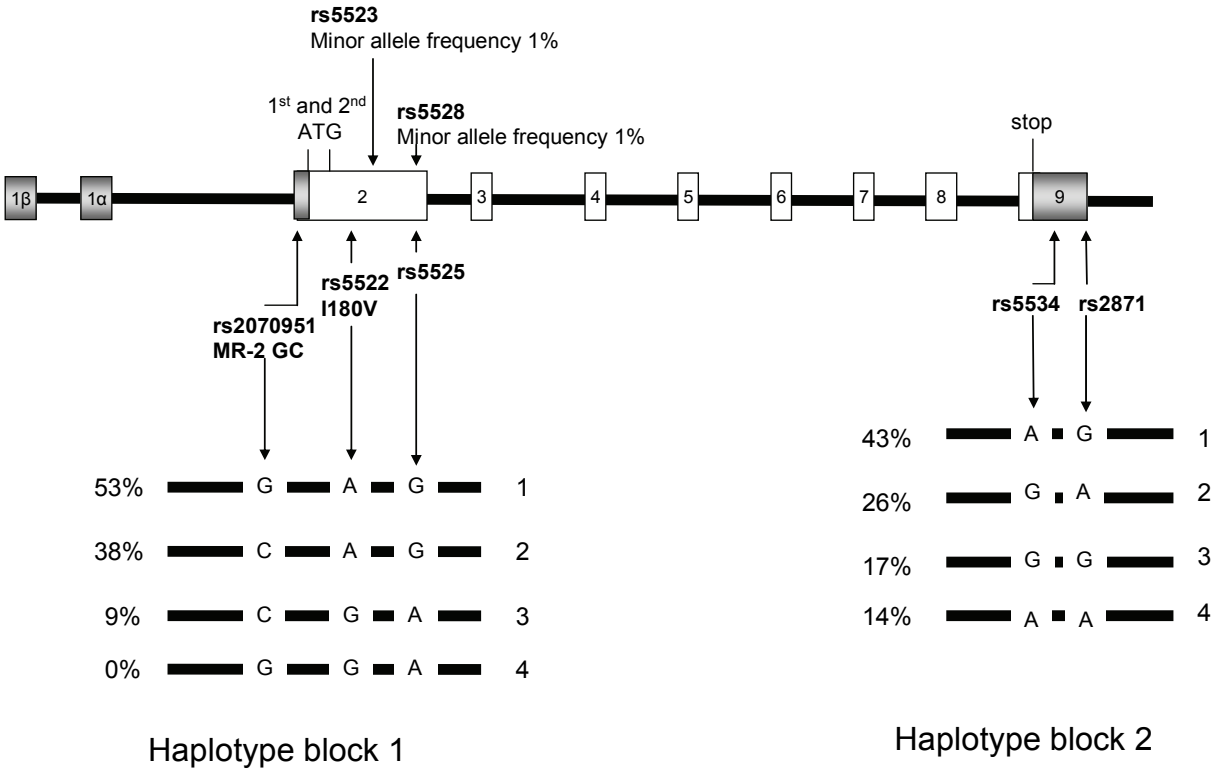


Figure 2. Structure and haplotypes of the hMR gene. Intronic DNA regions are displayed as the black line in the middle; exonic regions are displayed as the boxes on the line. Gray boxes represent the untranslated exons. White boxes indicate translated exons. SNPs and haplotypes are indicated below the DNA

Discussion

Screening the coding region of the MR gene revealed seven single nucleotide polymorphisms (SNPs), single nucleotide changes with a frequency of more than 1% in the population. No new SNPs were identified; these seven SNPs were already described on the NCBI website (http://www.ncbi.nlm.nih.gov/snp). There were no SNPs observed or previously described in the DNA and ligand binding domain. Genetic variation in these regions has probably too severe consequences for physiological processes to occur frequently in a healthy population. This assumption is supported by the finding that if rare genetic mutations occur in these regions, they invariably result in pseudohypoaldosteronism type I (PHA1).

Two haplotype blocks were identified in the MR. One containing the SNPs in exon 2, the other containing SNPs in exon 9. Block 1 contains the previously tested, *in vivo* and *in vitro* functional SNPs MR-2G/C and MRI180V. Functionality of haplotype block 2 or the separate SNPs in haplotype block 2 has not been reported but SNPs in this region might influence mRNA stability, since this has been demonstrated for other genes, including the closely related GR gene.

The frequencies of rs5523 and rs5528, two of the seven SNPs in the coding region, were very low, only 1%, therefore these SNPs can not be used for future association studies in small patient-control cohorts.

3

Functional Mineralocorticoid Receptor (MR) gene variation influences the cortisol awakening response after dexamethasone

Nienke van Leeuwen, Robert Kumsta, Sonja Entringer, E. Ronald de Kloet, Frans G. Zitman, Roel H. DeRijk & Stefan Wüst

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Summary

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA) axis and results in secretion of corticosteroids, which facilitate behavioral adaptation and promote the termination of the stress response. These actions exerted by cortisol are mediated by two brain corticosteroid receptor types: the high affinity mineralocorticoid (MR) and the lower affinity glucocorticoid receptor (GR). Dexamethasone is a potent GR agonist with affinity to MR. Administration of dexamethasone in the evening results in a significant suppression of the morning cortisol awakening response (CAR).

Here we tested the involvement of MR variants in this effect of dexamethasone in 218 young healthy subjects (125 females, all using oral contraceptives). For this purpose we determined two single nucleotide polymorphisms (SNPs) in the MR gene, the previously described MRI180V (rs5522) and the MR-2G/C (rs2070951), which both affect *in vitro* the transactivational capacity of the MR in response to either cortisol or dexamethasone.

Administration of a low dose dexamethasone (0.25mg) at 2300h resulted in a significant suppression of the cortisol awakening response (CAR). Both SNPs modulated the suppression of the CAR after dexamethasone significantly and in a sex specific manner. Suppression of the CAR was highest in the female MR-2G/C GG subjects while in male GG subjects the dexamethasone suppression of the CAR was attenuated compared to the MR-2G/C GC and CC groups. For the MRI180V, male AA subjects showed after dexamethasone a higher CAR than AG subjects while this effect was not observed in females. The SNPs had no significant influence on the CAR without prior dexamethasone treatment.

The association of the CAR with functional MR gene variants only in dexamethasone treated subjects suggests the involvement of MR in dexamethasone induced suppression of morning cortisol.

Key words: mineralocorticoid receptor, genetic variation, HPA axis, dexamethasone, cortisol

Introduction

Corticosteroids, secreted by the adrenals, have metabolic, immunological and cardiovascular effects and facilitate behavioural adaptation (de Kloet et al., 2005). As corticosteroid dysregulation is potentially harmful for the body, the secretion of corticosteroids as end product of the hypothalamic-pituitary-adrenal (HPA) axis is strictly regulated at several levels including the hippocampus, hypothalamus, pituitary and adrenals. Regulation of the HPA axis by cortisol is mediated in complementary fashion by two brain corticosteroid receptor types, the low affinity glucocorticoid receptor (GR, NR3C1) and the high affinity mineralocorticoid receptor (MR, NR3C2). The MR is substantially occupied under basal conditions and studies in rats suggest a role of MR in basal HPA activity (Ratka et al., 1989), particularly in feedback regulation of the pulsatile release of corticosteroids (Atkinson et al., 2008). In humans administration of a selective MR antagonist increases basal cortisol levels (Dodt et al., 1993; Born et al., 1997; Deuschle et al., 1998; Young et al., 1998; Heuser et al., 2000; Arvat et al., 2001; Otte et al., 2003a; Otte et al., 2003b; Wellhoener et al., 2004; Buckley et al., 2007). In most of these studies ACTH levels were unchanged or decreased.

The GR is mainly assumed to control feedback regulations after stress. The potent synthetic glucocorticoid dexamethasone is thought to act predominantly on the GR at the level of the pituitary gland to inhibit stress-induced HPA axis activation (De Kloet R et al., 1975). This steroid is commonly used in the dexamethasone suppression test (Carroll et al., 1968) as well as in the combined dexamethasone-CRH test. In both tests dexamethasone suppresses the HPA axis and the brain becomes depleted of the naturally occurring glucocorticoid cortisol. It is hypothesized that under those conditions the remaining low concentrations of cortisol occupy only brain MR and not GR. These tests have been frequently used to assess HPA axis regulation in psychiatric diseases. Patients with major depression do often show reduced cortisol suppression after a low dose of dexamethasone, whereas post traumatic stress disorder has been associated with an increased suppression after dexamethasone (Yehuda, 2002).

In the current study we addressed the question if the MR contributes to morning HPA axis activity and the effects of dexamethasone on this morning HPA axis activity by measuring saliva cortisol and plasma ACTH levels. There are two possibilities that may explain how the MR can influence this effect of dexamethasone. First, the residual MR occupancy after low dose dexamethasone treatment could control the drive from higher brain regions to the HPA axis (Holsboer, 2000). The second possibility is that dexamethasone, which has affinity for MR (Rupprecht et al., 1993; Grossmann et al., 2004) binds brain MR, but that this usually remains unnoticed because of the manifold higher potency of dexamethasone at the pituitary GR.

Two common functional MR gene variants, MRI180V and MR-2G/C were used to investigate the involvement of the MR on morning HPA axis activity with and without dexamethasone. Previously, we described the MR gene variant MRI180V (rs5522), a single nucleotide polymorphism (SNP) in exon 2, which results in an amino acid change in the N-terminal domain of the protein. This SNP decreases the transactivation capacity of the MR *in vitro* and was associated with enhanced cortisol and autonomic responses to an acute psychosocial stressor (DeRijk et al., 2006). In the current study

we tested a second MR gene variant called MR-2G/C, for its influence on the *in vitro* transactivation capacity with cortisol and dexamethasone as ligands. This SNP is located two nucleotides before the translation start site of the MR and might affect the translation of the MR protein.

We then tested if these two MR gene variants are associated with the cortisol awakening response (CAR) either with or without dexamethasone treatment. The CAR is a distinct rise in cortisol levels occurring in response to morning awakening (Wilhelm et al., 2007). This measure is increasingly used as an indicator of adrenocortical activity and, amongst others, the CAR was shown to be related to gender, depressive symptomatology, PTSD, primary insomnia, chronic fatigue as well as to neuroticism and perceived chronic stress (Wüst et al., 2000a; Wüst et al., 2000b; Bhagwagar et al., 2003; Pruessner et al., 2003; Backhaus et al., 2004; Roberts et al., 2004; Rohleder et al., 2004; Schlotz et al., 2004; Portella et al., 2005; Wessa et al., 2006). A moderate, yet significant heritability of the CAR was found (Wüst et al., 2000a; Wüst et al., 2000b; Bartels et al., 2003). In recent review papers a comprehensive overview of findings related to this measure and a discussion of potential mechanisms mediating the CAR can be found (Clow et al., 2004; Fries et al., 2008; Chida and Steptoe, 2009).

Thus, we investigated the association of the two MR gene variants with the CAR and early morning ACTH levels with and without dexamethasone administration.

Materials and methods

Subjects

In the present study we investigated 218 healthy subjects, 125 females and 93 males (mean age 25.1 years, standard error of mean (SEM) of ± 0.26 years; BMI 24.3 ± 0.28), who were recruited from a Trier-based community sample and from the student body of the University of Trier. All participants were non-smokers. Ethnicity was assessed by asking for the geographical origin of the subjects' parents and grandparents. Except some grandparents who originated from Poland, all subjects, parents and grandparents originated from Germany. Except for ethinyl-estradiol/progestin containing oral contraceptives (used by all females; see below for a discussion), all subjects reported to be medication free. Absence of acute or chronic diseases was confirmed in a medical examination prior to the experimental session. The protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants. The 218 subjects were initially selected for testing genetic variation in the GR (for details see Kumsta et al., 2007).

Cortisol awakening response (CAR)

Subjects were asked to collect saliva samples on two consecutive weekdays immediately after awakening and 30, 45, 60 minutes after the first sample using Salivette sampling devices (Sarstedt, Nuembrecht, Germany). They were instructed to refrain from food, drinks other than water and brushing their teeth before completion of saliva sampling.

Dexamethasone suppression test

Participants were instructed to ingest 0.25mg dexamethasone (Par Pharmaceutical, Spring Valley, USA) at 2300h and to collect saliva samples as described above for the assessment of the CAR after dexamethasone ingestion. Moreover, one EDTA blood sample was obtained to assess ACTH and dexamethasone levels when the subjects reported to the laboratory 60 to 90min after awakening between 0800 and 0830h.

Biochemical analyses

Saliva samples were stored by the subjects in their freezers and returned to the laboratory after completion of sampling. Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendorfer et al., 1992). EDTA blood samples were immediately stored on ice and centrifuged within 30min at 2000 x g and 4°C for 10min. Plasma was stored at -80°C until analysis. ACTH was measured in EDTA plasma with a chemoluminescence immunoassay (Nichols institute, Bad Nauheim, Germany). Plasma dexamethasone was assessed with an in-house RIA at the Institute of Pharmacology, University of Heidelberg. Inter-assay and intra-assay coefficients of variance were below 10% and 12%, respectively, for all analyses.

DNA extraction and genotyping

DNA was extracted from 10ml peripheral venous blood following a standard NaCl salting out method (Miller et al., 1988). All subjects were genotyped for the MR-2G/C and MRI180V SNPs in duplicate using TaqMan pre-designed SNP genotyping assays, assay ID C12007869_20 and C1594392_10 respectively, in combination with TaqMan universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Reaction components and amplification parameters were based on the manufacturer's instructions. In addition, all the samples were genotyped with direct sequencing, therefore a PCR reaction was performed with 50ng DNA, 8.0pM of each primer (Forward: TCGCTTCTCTTGTTCT GACA, Reverse: CTGGACTCATGCTTCCTTGT) and AmpliTaq Gold (Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands), according to the manufacturer's protocol. Sequencing of the PCR product was done with BigDye Terminator Sequencing Kit v3.1(Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands) on an ABI Prism 3100-Avant Genetic Analyzer, according to the manufacturer's instructions, while sequences were analyzed using Vector NTI (Invitrogen).

Site directed mutagenesis of hMR

The recombinant pRSV human MR plasmid, containing the last 30 base pairs of exon 1α into the nontranslated region of exon 9, was obtained from Dr. R. Evans (gene expression laboratory and HHMI, The Salk Institute for Biological Studies, La Jolla, Ca). The rs2070951 site was mutated from G to C with the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the primers 5'-GGCCGAGGCAGCGATGGAGACCAAAG-3' and 5'-CGCTGCCTCGGCCCTTTGGTCTCCAT-3' according to the manufacturers protocol. After mutagenesis the hMR insert of the plasmid was sequenced to assure absence of other mutations. Plasmids were purified from DH5α E-coli bacterial cultures using the Pure Yield purification system (Promega, Leiden, The Netherlands).

Transfections and luciferase assays

Cos-1 cells (African green monkey kidney cells) were cultured in DMEM high glucose supplemented with 10% FCS (Gibco, Paisley, UK). Cells were seeded in 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 3 x 10^4 cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using SuperFect (Qiagen, Venlo, The Netherlands). Plasmids containing one of the hMR variants or no hMR (control) and the reporter plasmid TAT3-Luc (tyrosine amino transferase triple hormone response element) were used at 100 ng/well. The control plasmid pCMV-R (Promega, Leiden, The Netherlands) coding for Renilla luciferase controlled by cytomegalovirus (CMV) promoter was used (10 ng/well) for normalization purposes. One day after transfection, the cells were treated with either cortisol (Sigma-Aldrich, Zwijndrecht, the Netherlands) or dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands) in the concentrations 0, 10^{-11}, 3.10^{-11}, 10^{-10}, 3.10^{-10}, 10^{-9} and 10^{-8} M. After 24h of incubation the cells were harvested in passive lyses buffer (Promega) and firefly and Renilla luciferase activity was determined using the dual label reporter assay (Promega) and a luminometer (CENTRO XS3 LB960, Berthold, Bad Wildbad, Germany).

Statistical analyses

In the *in vitro* assays the firefly/renilla luciferase ratios were normalized to both the lowest and highest luciferase ratio for each hMR variant, background expression was subtracted and differences between the hMR variants were analyzed with a two-way ANOVA with Bonferroni posttest and by comparing the EC50 and slope of the dose response curves with a t-test using GraphPad prism 4 (GraphPad software Inc, San Diego, CA). Haploview (Barrett et al., 2005) was used to calculate Hardy Weinberg equilibrium (HWE) and linkage disequilibrium among the two MR SNPs (estimated with D' and r^2). To test if the MR genotypes are equally distributed in the different GR genotype groups a X^2 test was computed. Cortisol data were log transformed to yield unskewed outcome variables. The two CAR measurements (without prior dexamethasone administration) were averaged in order to enhance the reliability of the measure (Wüst et al., 2000b; Hellhammer et al., 2007). General linear models (GLMs) were computed to assess the repeated measures effect *time*, the between-subjects effect *genotype* as well as the interaction *time x genotype* for the CAR with and without dexamethasone pretreatment. In order to reveal *sex x genotype* interactions, sex was also included as predictor in the GLMs. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. One-way ANOVAs were performed to compare mean plasma dexamethasone and ACTH levels after dexamethasone administration between comparison groups. All results shown are the mean ± SEM unless otherwise stated. Apart from the exceptions stated above statistical analyses were performed using SPSS 14.0 (Chicago, IL, USA). While statistical analyses of cortisol levels were based on log-transformed data, figures show untransformed means in order to provide a more naturalistic impression of cortisol levels.

Results

In vitro functionality testing

Cos-1 cells were transfected with plasmids containing either no MR, the MR-2C or MR-2G nucleotide. Each plasmid was three times cultured, purified and tested to exclude variation due to

impurity or concentration of the plasmid. In each of the three experiments the plasmids were tested in triplicate. After incubation with either cortisol or dexamethasone at 7 concentrations in the range from 0 to 10^{-8}M luciferase activity was measured. The negative control, cells transfected with plasmids containing no MR, showed only with the highest (10^{-8}M) dexamethasone concentration an induction of 12%. For the other dexamethasone concentrations and all the cortisol concentrations there was no induction of luciferase in the negative control. After normalization of the luciferase data the 12% background induction was subtracted.

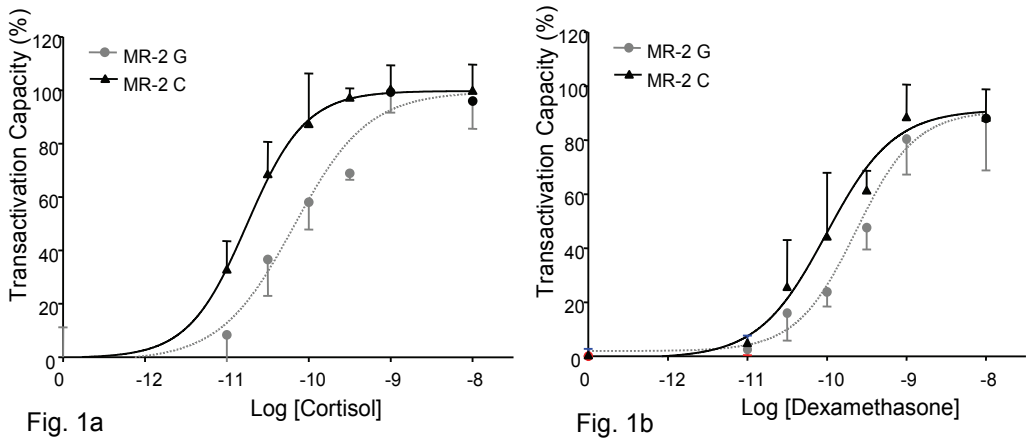

Figure 1 a) Cortisol and b) dexamethasone driven transactivation capacity of the MR -2G/C variants on a TAT-3 promoter in Cos-1 cells displayed as percentage of the maximal transactivation capacity (±SD) – the background activity when no MR is present. Cortisol and dexamethasone concentrations are indicated in log units. The MR-2G variant (dashed circle) showed a lower transactivation capacity compared with the MR-2C variant (black triangle) (cortisol $p<0.001$; dexamethasone $p<0.01$).

The MR-2C variant showed significantly higher transcriptional activity than the MR-2G variant using either cortisol or dexamethasone ($F_{1,86}=52.3$, $p<0.001$ for cortisol and $F_{1,86}=7.3$, $p=0.01$ for dexamethasone, Figure 1a & b). Post hoc analysis showed significant genotype effects at cortisol concentrations of 3.10^{-11}, 10^{-11}, 10^{-10} and 3.10^{-10}M ($t=4.265$, $t=5.770$, $t=5.297$, all $p<0.001$ and $t=3.11$ with $p<0.05$, respectively) and at a dexamethasone concentration of 10^{-10} ($t=3.26$ and $p<0.05$). Analysis of the dose response curves showed a significant difference in the EC50 between the two MR variants (cortisol $t_{14}=5.816$, $p<0.0001$ and dexamethasone $t_{14}=2.747$, $p=0.016$) but no difference in the slope of the curves. For cortisol the EC50 was $6.6.10^{-11}$ for MR-2 G and $1.7.10^{-11}$ for MR-2 C and for dexamethasone the EC50s were $2.3.10^{-10}$ and $9.9.10^{-11}$ respectively.

Genotyping

The 218 subjects were genotyped for the polymorphisms MRI180V and MR-2G/C with a Taqman assay and by direct sequencing yielding identical genotype frequencies (Table 1). Observed allele frequencies corresponded to those previously reported (DeRijk et al., 2006; Kuningas et al., 2007). While the MRI180V SNP was in Hardy Weinberg equilibrium (HWE) a deviation from HWE was detected for the MR-2G/C ($p=0.0153$; see below for a discussion). Estimated linkage between MRI180V and MR-2G/C was D'=1 (conf bounds 0.76-1) and $r^2=0.114$. Although the genes for the GR and the MR are located on different chromosomes (5q31.3 vs 4q31.1) a X^2 test was computed

confirming that the investigated MR SNPs were not statistically related to the GR SNPs that were determined in the same cohort ($X^2_{(20)}$=19.72, p=0.475; see Kumsta et al., 2007).

Table 1
Observed genotypes

	MRI180V		∑
	AA	**AG**	
MR-2 CC			
Male	12	6	18
Female	17	13	30
Total	29 (13.3%)	19 (8.7%)	48 (22.0%)
MR-2 CG			
Male	43	11	54
Female	59	15	74
Total	102 (46.8%)	26 (11.9%)	128 (58.7%)
MR-2 GG			
Male	21	0	21
Female	21	0	21
Total	42 (19.3%)	0 (0%)	42 (19.3%)
∑	173 (79.4%)	45 (20.6%)	**218 (100%)**

Cortisol awakening response (CAR) with and without prior dexamethasone administration
As previously described the CAR differed significantly between males and females, with males showing higher responses. Therefore sex was included as a predictor in all further analyses. The CAR without prior dexamethasone treatment showed the expected mean rise. However, we did not find any significant association between MR genotypes or haplotypes and the cortisol increase after awakening. The only noteworthy statistical trends were the interaction *genotype x sex* ($F_{2,204}$=1.62, p=0.20) for the MR-2G/C (Fig. 2b) and the interaction *time x genotype x sex* ($F_{1.98,\ 408.6}$=2.38, p=0.09) for MRI180V (Fig. 2a), suggesting a somewhat higher CAR in male MRI180V AA subjects (see also Table 2 for an overview of effects).

The assessment of plasma dexamethasone levels confirmed that all participants had ingested the dexamethasone tablet and no significant difference between males and females was observed (males: 168 ± 51, females 174 ± 40 ng/100 ml, $F_{2,212}$=.863, p=0.4). Moreover, individual dexamethasone levels were not associated with the MR genotypes (all F<1.05 and all p>.39). After 0.25mg dexamethasone the CAR was still observed but cortisol concentrations were significantly lower then without prior dexamethasone treatment (main effect *treatment:* $F_{1,189}$=597.8, p<.001). The average starting level was reduced from 11.2 ± 5.1 to 2.9 ± 1.8 nmol/l (74% suppression) and the average peak level from 17.0 ± 6.9 to 4.5 ± 2.3 nmol/l (74% suppression). Furthermore, cortisol levels in females (all using OCs) were significantly lower than in males (main effect *sex:* $F_{1,196}$=36.69, p<.001)

Both MR SNPs were associated with post-dexamethasone cortisol levels, measured as the CAR, in a sex specific way (see Fig. 3a & b). In males MRI180V AA subjects (homozygotes major allele) showed a clearly higher (i.e. less suppressed) mean CAR than MRI180V G carriers, while this effect could not be observed in females resulting in a significant *genotype x sex* interaction ($F_{1,193}$=4.34, p=0.039). Also independent of the subjects' gender we found a trend towards a main effect *genotype* ($F_{1,193}$=2.84, p=0.093) but this trend was obviously driven by the large difference in males. Accordingly, this main effect *genotype* was significant when females were excluded in a supplementary analysis ($F_{1,80}$=5.06, p=0.027). For the MR-2G/C an even clearer sex specific effect was detected with GG subjects showing the largest CAR in males and the most suppressed CAR in females (interaction *genotype x sex*: $F_{2,191}$=4.23, p=0.016).

As MRI180V and MR-2G/C are in linkage disequilibrium an additional analysis based on haplotypes was performed (see Fig. 3c). Three haplotypes were detected, MR-2-G/I180V-A, MR-2-C/I180V-A and MR-2-C/I180V-G and the results of the haplotype analysis confirmed the genotype based comparisons. A significant *genotype x sex* interaction was detected *(*$F_{4,187}$=2.84, p=0.026) and again this interaction was primarily based on the difference between male and female MR-2G/C GG subjects. A remarkable comparison group in this analysis were the male MR-2CC/I180V-AG subjects who showed a virtually completely suppressed CAR (see also Table 2 for an overview of effects).

Table 2. General linear model effects for the CAR with and without dexamethasone pre-treatment

		CAR			CAR DEX		
	Effect	**df**	**F**	**p**	**df**	**F**	**p**
MR-2G/C	Genotype	2;204	1.53	.220	2;191	0.26	.974
	Sex	1;204	2.14	.145	1;191	37.18	<.001
	Genotype x Sex	2;204	1.62	.200	2;191	4.23	.016
	Time	1.96;400.73	77.57	<.001	2.15;410.01	66.89	<.001
	Time x Genotype	3.93;400.73	0.70	.593	4.29;410.01	1.36	.244
	Time x Sex	1.96;400.73	16.90	<.001	2.15;410.01	10.07	<.001
	Time x Genotype x Sex	3.93;400.73	0.68	.601	4.29;410.01	1.57	.179
MRI180V	Genotype	1;206	0.56	.457	1;193	2.84	.093
	Sex	1;206	0.31	.575	1;193	10.94	.001
	Genotype x Sex	1;206	0.67	.414	1;193	4.34	.039
	Time	1.98;408.56	71.13	<.001	2.16;416.02	49.14	<.001
	Time x Genotype	1.98;408.56	0.49	.610	2.16;416.02	1.83	.158
	Time x Sex	1.98;408.56	8.61	<.001	2.16;416.02	3.63	.024
	Time x Genotype x Sex	1.98;408.56	2.38	.094	2.16;416.02	0.98	.38
Haplotypes	Genotype	4;200	1.30	.270	4;187	1.02	.40
	Sex	1;200	0.66	.416	1;187	15.01	<.001
	Genotype x Sex	4;200	0.93	.448	4;187	2.84	.026
	Time	1.98;395.98	69.03	<.001	2.14;399.94	48.51	<.001
	Time x Genotype	7.92;395.98	0.77	.625	8.56;399.94	1.60	.117
	Time x Sex	1.98;395.98	9.55	<.001	2.14;399.94	3.82	.020
	Time x Genotype x Sex	7.92;395.98	1.13	.340	8.55;399.94	1.29	.244

ACTH levels, assessed between 0800h and 0830h after dexamethasone administration were significantly more suppressed in females (all using OCs) than in males (main effect *sex:* $F_{1,213}$=21.56, p<.001). We did, however, not detect any significant association between ACTH levels and MR genotype (MRI180V: means±SEM: 31.18±1.80 for AA, 35.35±5.33 for AG; main effect *genotype:* $F_{1,212}$=0.95, p=0.33; interaction *genotype x sex*: $F_{1,212}$=1.28, p=0.26; MR-2G/C: means±SEM: 28.68±4.23 for CC, 33.06±2.94 for GC, 32.68±5.17 for GG; main effect *genotype:* $F_{2,212}$=0.17, p=0.84; interaction *genotype x sex*: $F_{2,212}$=0.56, p=0.95; haplotypes: means±SEM: 32.02±6.05 for CC/AA, 30.36±3.02 for GC/AA, 32.68±5.17 for GG/AA, 23.49±5.54 for CC/AG, 43.57±8.54 for GC/AG; main effect *genotype:* $F_{4,212}$=1.29, p=0.27; interaction *genotype x sex*: $F_{4,212}$=0.24, p=0.92).

Figure 2 (next page) Mean cortisol awakening responses (± SEM) in males and females with the MR genotype **(a)** I180V AA (males n= 76, females n= 97) and I180V AG (males n= 17, females n= 28); **(b)** -2CC (males n= 18, females n= 30), -2GC (males n= 54, females n= 74) and -2GG (males n= 21, females n= 21); **(c)** MR haplotypes -2CC/ I180V AA (males n= 12, females n=17), -2GC/ I180V AA (males n= 43, females n= 59), -2GG/ I180V AA (males n= 21, females n=21), -2CC/ I180V AG (males n= 6, females n= 13) and -2GC/ I180V AG (males n= 11, females n= 15).

Figure 3 (next page) Mean cortisol awakening responses (± SEM) after 0.25mg dexamethasone at 2300h the evening before in males and females with the MR genotype **(a)** I180V AA (males n= 76, females n= 97) and I180V AG (males n= 17, females n= 28) ; **(b)** -2CC (males n= 18, females n= 30), -2GC (males n= 54, females n= 74) and -2GG (males n= 21, females n= 21); **(c)** MR haplotypes -2CC/ I180V AA (males n= 12, females n=17), -2GC/ I180V AA (males n= 43, females n= 59), -2GG/ I180V AA/ (males n= 21, females n=21), -2CC/ I180V AG (males n= 6, females n= 13) and -2GC/ I180V AG (males n= 11, females n= 15). Please note the different y-axes scales in Fig. 2 and 3.

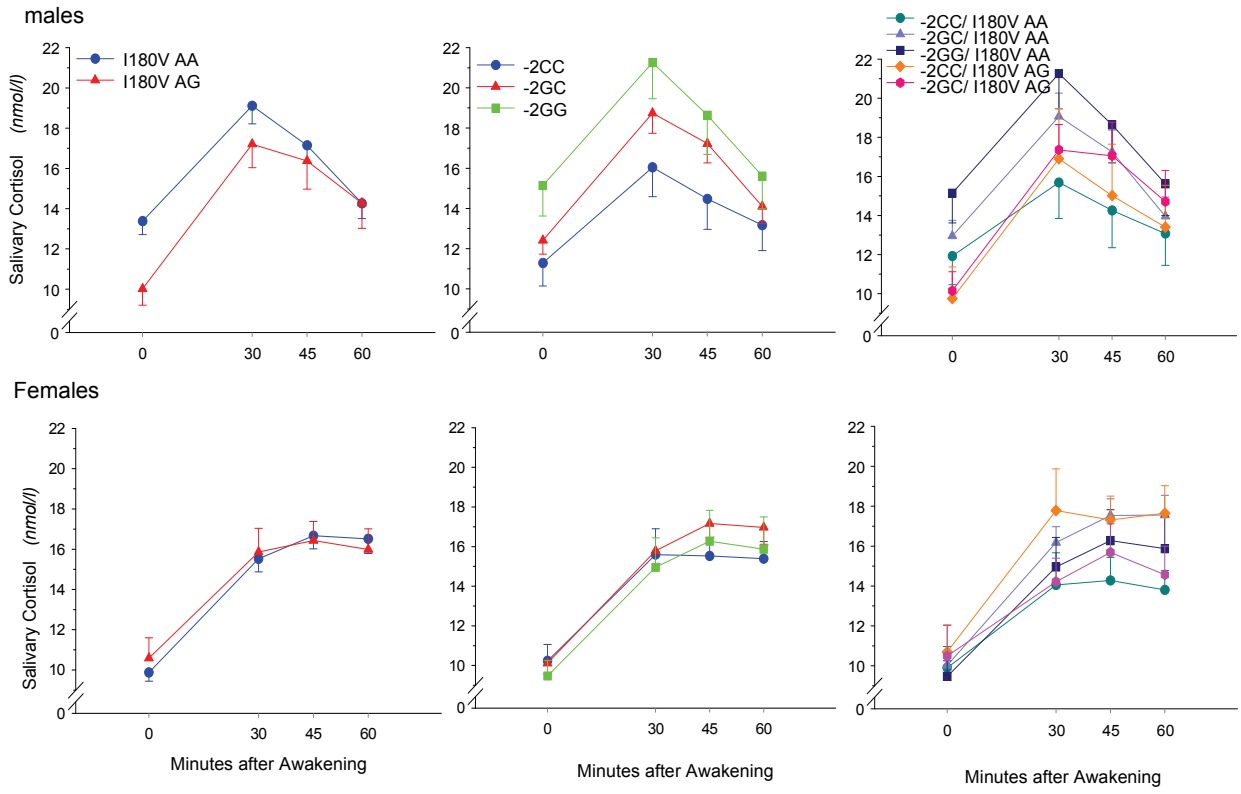
males
I180V AA
I180V AG
-2CC
-2GC
-2GG
-2CC/ I180V AA
-2GC/ I180V AA
-2GG/ I180V AA
-2CC/ I180V AG
-2GC/ I180V AG
Females
Salivary Cortisol (nmol/l)
Minutes after Awakening

Fig. 2

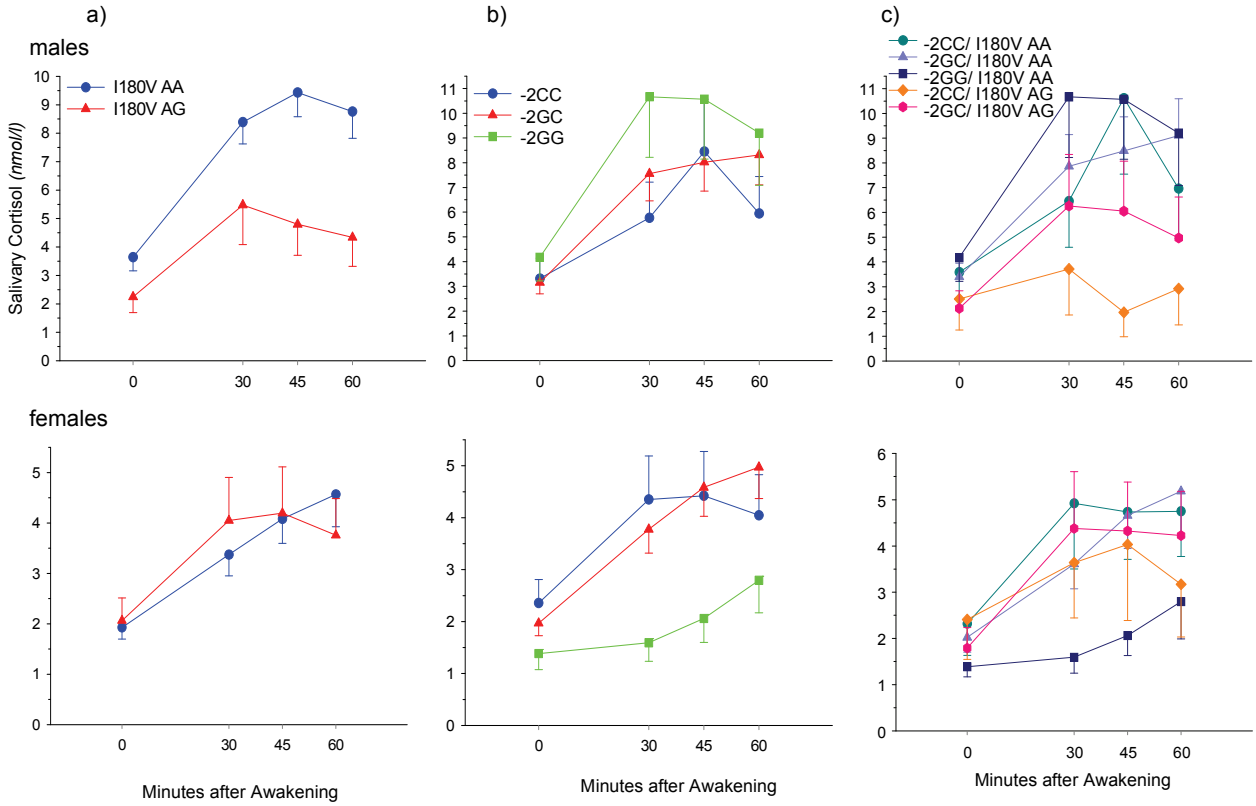
a)
b)
c)
males
females
I180V AA
I180V AG
-2CC
-2GC
-2GG
-2CC/ I180V AA
-2GC/ I180V AA
-2GG/ I180V AA
-2CC/ I180V AG
-2GC/ I180V AG
Salivary Cortisol (nmol/l)
Minutes after Awakening

Fig 3.

Discussion

Two *in vitro* functional MR gene variants, MRI180V and MR-2G/C were associated with suppression of the cortisol awakening response following administration of 0.25mg dexamethasone. This effect was sex specific and was also observed with the haplotype of both SNPs. Moreover, when males were analyzed separately, a main effect for the MRI180V was observed. However, neither the two SNPs nor the haplotype were significantly related to awakening cortisol responses without prior dexamethasone treatment.

Previously, we showed that the minor allele of the MR180V results in a relative loss of function *in vitro* using cortisol as ligand and although we did not show the data, we observed this as well with our assay. The present study demonstrates that the MR-2C variant has a significantly higher capacity in the activation of TAT-3 regulated gene transcription, as compared to the MR-2G, using either cortisol or dexamethasone as ligands. MR-2G/C is located two nucleotides before the first translation startsite in the kozak region, which is important for translation of the MR. The MR contains two translation start sites resulting in the isoforms MR-A and MR-B (Pascual-Le Tallec et al., 2004). MR-A is more potent in activating a MMTV or a GRE containing promoter than MR-B when aldosterone is used as a ligand. The wild type plasmid, expressing both MR-A and MR-B, has an even higher transactivation capacity than MR-A. Therefore, it can be hypothesized that the ratio MR-A/ MR-B might be important. This MR-2G/C SNP might change this MR-A / MR-B ratio and thereby the transactivation capacity. The observed effects of the studied SNPs *in vitro* indicate that they can be functional *in vivo*, although the sex specific effects observed in the association study indicate that a precise prediction of *in vivo* consequences from *in vitro* data is very difficult. On the other hand, these functional genetic variants provide natural tools to study the involvement of the MR in HPA axis regulation.

Dexamethasone activates GR exerting long lasting genomic effects on negative feedback. This occurs predominantly at the pituitary level and it causes suppression of HPA axis activity until the next morning. However, the differential effects of functional MR gene variants on dexamethasone suppression of cortisol levels indicate the involvement of the MR. Several mechanisms can account for this observation.

First, the MR gene variants, as *in vitro*, are expected to react differently *in vivo* to stimulation with dexamethasone and thus also differentially affect HPA axis suppression. This is a likely explanation, since without dexamethasone there was no genotype effect on the CAR. Moreover, dexamethasone has an appreciable affinity fir the MR (Rupprecht et al., 1993; Grossmann et al., 2004). Although likely, a mechanism assuming direct MR binding of dexamethasone needs further investigation. Central brain MR are less accessible for dexamethasone since Pg-p hampers the penetration of the steroid into the brain (Meijer et al., 1998). In spite of this some dexamethasone might pass the blood brain barrier and activate MR, while the pituitary gland also contains some MR.

Second, 0.25mg dexamethasone results in lower, but still appreciable levels of saliva cortisol (Fig. 3). The levels are comparable with the levels normally observed in the afternoon and are probably sufficient to activate MR (Meijer et al., 1998; Karssen et al., 2005; Droste et al., 2008). It is

hypothesized that under these conditions the differential effects of the MR gene variants appear, with subsequent effects on the input from higher limbic brain regions on the hypothalamic AVP (and/or CRH) drive to pituitary ACTH release (Bradbury et al., 1994; Tajima et al., 1999; Kovacs et al., 2000). Since limbic MR has inhibitory effects on the HPA axis, a reduced MR activation may result in this enhanced drive (Holsboer, 2000). However, we did not observe a significant association with ACTH levels collected between 0800h and 0830h, the reason for this inconsistency is not clear. As the subjects awoke at least one hour prior to blood sampling the ACTH measures reflect hormonal levels after the CAR. It could thus be speculated that an association with the MR genotype can be detected when the awakening response (cortisol or ACTH) is assessed but not when single early morning levels are considered.

Finally, fast non-genomic actions of membrane bound MR have been described. This membrane bound MR has much lower affinity for corticosterone then the classic MR with its genomic actions (Karst and Joels, 2005; Joels et al., 2008) and was recently shown in rats to mediate fast feedback during the ultradian pulse (Atkinson et al., 2008). These membrane bound MR have low affinity for corticosteroids and require rising corticosteroid levels for activation which makes a role for this non-genomic mechanism less likely.

Changes in cortisol suppression after dexamethasone have been associated with psychopathology such as PTSD and major depression. The involvement of MR variants in the dexamethasone induced suppression of the CAR is an indication that those MR variants might be involved in psychopathology as well. A previous study by Kuningas et al supports this hypothesis since they found an association between MRI180V and feelings of depression in a cohort consisting of elderly (Kuningas et al., 2007). However, we used the CAR as a more precise measure of dexamethasone induced suppression of cortisol instead of one cortisol sample taken in the morning. The functionality of the CAR (with or without dexamethasone administration) for pathology is not completely understood yet, making it difficult to draw more far-reaching conclusions.

Sex differences in HPA axis basal activity, regulation and reactivity are well known (Kudielka and Kirschbaum, 2005). Our previous study using the same cohort revealed a sex specific association between the GR *Bcl*I SNP and cortisol responses to a psychosocial stressor (Kumsta et al., 2007). Moreover, the GR SNP 9βAG was significantly associated with morning ACTH levels after dexamethasone administration, but only in males. In the current study, the association with post dexamethasone cortisol concentrations in males seems to be due to the MRI180V SNP. In contrast, the MR-2G/C SNP had the largest effect in females with the MR-2GG group showing a suppressed CAR. Thus it seems that the different variants of the MR gene can have opposite effects in males and females.

Since the MR has high affinity for aldosterone, cortisol, deoxycortisol and progesterone (Rupprecht et al., 1993), female hormones may interfere with actions mediated by cortisol. For example, expression of the MR is influenced by estrogens, while progesterone affects the binding of corticosterone in rat hippocampus (Carey et al., 1995). In rats, estrogens decrease the expression of the MR in the pituitary (Turner, 1990) and progesterone treatment increased the activity of the MR promoter in cell lines (Castren et al., 1995). Interestingly, after adrenalectomy in rats testosterone

rather then corticosterone regulates AVP expression (Viau et al., 1999). Furthermore, we recently showed that MRI180V interacts differentially with different ligands. While the MRI180V modulates cortisol dependent transactivation *in vitro*, no difference was observed with aldosterone as a ligand (DeRijk et al., 2006). Thus both unique ligand – MR gene variant interactions and sex hormone modulation of MR gene expression could underlie the gender specific interactions of MR gene variants on post-dexamethasone cortisol levels.

It is a certain limitation of our study that we could not control for the subjects' compliance with the saliva sampling protocol. In studies that used collection devices equipped with electronic sensors it was revealed that strict compliance with instructions cannot be granted in ambulatory settings (Kudielka et al., 2003; Broderick et al., 2004). However, such an inaccuracy usually affects only a relatively small proportion of measurements, on average the ambulatory CAR is identical with the CAR assessed under sleep laboratory conditions (Wilhelm et al., 2007) and – most importantly - it is extremely unlikely that possible sampling time inaccuracies are systematically associated with our MR genotype groups.

Another limitation of our study concerns the interpretability of the observed sex by genotype effects since all female participants used oral contraceptives (OCs). We chose to investigate only oral contraceptive using women as the complete study protocol included the exposure to a laboratory stress protocol (for details see (Kumsta et al., 2007) and in order to avoid a modulation of cortisol and ACTH responses to acute stress by the menstrual cycle phase (Kirschbaum et al., 1999).

It is well known that ethinyl-estradiol leads to increased corticosteroid binding globulin (CBG) synthesis, which in turn influences total serum cortisol levels and the availability of free cortisol. Indeed, CBG was shown to modulate hormonal responses to TSST exposure in oral contraceptive using females, as well as in males (Kumsta et al., 2007). However, the CAR (without dexamethasone pretreatment) was found to be not significantly affected by OCs in a relatively large cohort of 509 subjects (319 females, 29% used OCs) (Wüst et al., 2000b).

Data on a potential influence of OCs on HPA axis hormones after dexamethasone administration are surprisingly scarce and we cannot rule out such an effect in our study. However, after 1mg dexamethasone at 2300h free cortisol levels at 1600h did not significantly differ in women taking contraceptives compared to women in various stages of the menstrual cycle (Ansseau et al., 1993). Furthermore, at least the absolute levels of CBG per se are likely not responsible for our observed genotype by sex interactions, since we did not observe different CBG levels in the MR genotype groups (data not shown).

It could be argued that different OC brands differing in compositions could have differential effects on our measures. However, it appears rather unlikely that the usage of different OC brands was a major confounder in the present study. Females in our sample used 14 different OC brands (all monophasic) with 11 different compositions and we did not even detect a weak trend for a significant association between OC composition and the CAR, the CAR after dexamethasone or ACTH levels after dexamethasone administration. All main effects and interactions had a p value >.38. As most OC preparations contain .03mg ethinyl-estradiol we performed a further

supplementary analysis and compared females using OCs with .03mg, with less than .03mg and with more than 0.03mg ethinyl-estradiol. Again, we did not detect a significant effect on our measures (lowest p value: .25).

Generalization of our findings on women not taking oral contraceptives is not possible and this is a clear weakness of our study, which was accepted on the basis of feasibility considerations when the study was designed. It should be noted though, that more than 60% of young females (16-30 yrs) in Germany use OCs (von Have et al., 2005) and similar frequencies can be found in other industrialized countries. We thus think that studying OC females is a relevant topic.

The two investigated SNPs in the MR, MRI180V and MR-2G/C showed linkage disequilibrium (D'=1 (conf bounds 0.76-1) and r^2=0.114). This observed linkage has implications for the analysis because a combination of SNPs, a so called haplotype, can respond differently compared to isolated SNPs. Again a sex by genotype interaction was found for the association with post dexamethasone cortisol levels. Females with the MR-2/I180V GG/AA haplotype showed the lowest CAR, with no significant rise in cortisol at all, while men with this haplotype had the highest CAR. No unexpected interaction of the two SNPs, MRI180V & MR-2G/C, making the haplotypes was observed; both male MRI180V AG and MR-2G/C CC carriers showed lowest cortisol levels as well as the haplotype MRI180V AG/MR-2CC. In females the MR-2G/C made the difference, in contrast to the MRI180V, with its effect coming back in the haplotype, with the MR-2GG showing lowest cortisol levels. However, due to the rather small size of the haplotype groups these observations should be interpreted with caution. Subjects with MR-2 GG/MRI180V AG or MR-2 GG/MRI180V GG genotype combinations were not observed in this population and as part of a different study we sequenced several hundreds additional subjects and again these genotype combinations were not found (unpublished). This is reflected in the LD that was observed between the two SNPs.

The MR-2G/C SNP deviated from Hardy Weinberg equilibrium in the current cohort. This is often a result of a genotyping error but in this study we used two different methods for genotyping and found identical results, excluding a technical error. Furthermore, all individuals reported to be from central European descent making population stratification unlikely. The remaining explanations are random sample variation or the non-random selection of the cohort. The cohort was selected based on GR polymorphisms. The genes for the GR and the MR are located on different chromosomes (5q31.3 vs 4q31.1) therefore it is not expected that this selection influences the MR genotype distribution and indeed a X^2 test confirmed that the investigated MR SNPs were not statistically related to the GR SNPs. We only compared the genotype and haplotype effects on cortisol within this group and we did not perform a case-control study with a comparison between groups. In addition, the genotypes and haplotypes were used in the analysis instead of alleles. Therefore it is unlikely that this deviation from HWE had a major confounding impact on the findings. (Minelli et al., 2008).

In summary, the data suggest a role of the MR in the control of negative feedback of the HPA axis as tested with dexamethasone, but how the MR variants precisely modulate this feedback action remains to be established. The observed effects of these common functional variants of the human MR gene are gender specific.

4

The functional c.-2G>C variant of the Mineralocorticoid receptor modulates blood pressure, renin and aldosterone levels

Nienke van Leeuwen, Massimiliano Caprio, Carolina Blaya , Frédéric Fumeron, Paola Sartorato, Gilberta Giacchetti, Franco Mantero, Fabio Luiz Fernandes-Rosa, Christophe Simian, Sévrine Peyrard, Frans G. Zitman, Brenda W.J.H. Penninx, E. Ron de Kloet, Michel Azizi, Xavier Jeunemaitre, Roel H. DeRijk & Maria-Christina Zennaro

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Abstract

Context: The mineralocorticoid receptor (MR) is essential in the regulation of volemia and blood pressure. Rare mutations in the MR gene cause type 1 pseudohypoaldosteronism and hypertension.
Objective: Characterize the common MR polymorphism c.-2G>C (rs2070951) in vitro, and test its influence on parameters related to blood pressure regulation and the renin-angiotensin system.
Design: In vitro studies were performed to assess the influence of the polymorphism on MR expression and transcriptional activity. Association studies were performed with several outcome variables in three independent cohorts: a mild hypertensive group subjected to a salt sensitivity test, a healthy normotensive group included in a crossover study to receive both a high and low Na/K diet and a large cohort (the Netherlands Study of Depression and Anxiety (NESDA)), in which blood pressure was measured.
Results: The G allele was associated with decreased MR protein levels and reduced transcriptional activation compared to the C allele. Subjects with the GG genotype had significant higher plasma renin levels both in the mild hypertensive group and in normal volunteers compared to homozygous C carriers. The GG genotype was also correlated with higher plasma aldosterone levels in healthy subjects. In both the mild hypertensive group and the NESDA cohort the genotype GG was associated with higher systolic blood pressure in males.
Conclusions: The G allele of the common functional genetic polymorphism c.-2G>C in the MR gene associates with increased activation of the renin-angiotensin-aldosterone axis and with increased blood pressure, probably related to decreased MR expression.

Keywords: mineralocorticoid, aldosterone, hypertension, nuclear receptor, sodium balance

Introduction

The mineralocorticoid receptor (MR) mediates aldosterone effects on electrolyte balance and blood pressure (BP). Sodium handling is highly variable between individuals and genetic factors are involved in the development of hypertension (Halushka et al., 1999). Genetic variation in the MR might be responsible for the variability in sodium handling and vulnerability for hypertension. Classical MR-expressing tissues include the distal parts of the nephron, colon, salivary and sweat glands. In these tissues the MR regulates trans-epithelial sodium transport. However, MR are also expressed in non-epithelial tissues, including the cardiovascular system, the central nervous systems and adipose tissue (Caprio et al., 2007); in these tissues, glucocorticoids represent the predominant endogenous ligand (Funder, 2009).

The MR belongs to the nuclear receptor superfamily and acts as a ligand-activated transcription factor regulating expression of a coordinate set of genes ultimately eliciting physiologic aldosterone and cortisol responses. The gene coding for the human MR, NR3C2, is composed of 10 exons and spans over ~400 kb. By means of alternative promoter utilization, alternative splicing, use of different translational start sites and genetic polymorphisms, considerable variability in MR function has been observed (Pascual-Le Tallec and Lombes, 2005)(Zennaro et al., 1995). Rare mutations of the MR are responsible for Mendelian diseases characterized by disorders of renal salt handling associated with high or low BP. Loss of function mutations of the MR lead to type I pseudohypoaldosteronism (Pujo et al., 2007; Fernandes-Rosa et al., 2006; Sartorato et al., 2004; Riepe et al., 2003; Geller et al., 1998), while the rare activating mutation S810L (rs41511344) leads to juvenile hypertension exacerbated by pregnancy (Fagart et al., 2005; Pinon et al., 2004). Two recent studies showed associations between more common genetic variations, single nucleotide polymorphisms (SNPs), in the MR and BP (Martinez et al., 2009; Tobin et al., 2008). Previously we tested the amino acid changing SNP in exon 2, MRI180V (rs5522) *in vitro* and showed that the rs5522 G allele leads to a lower transactivational capacity (DeRijk et al., 2006). However, in a group of mild hypertensive individuals we did not find an association with BP and MRI180V (DeRijk et al., 2006) , and the frequency of the same polymorphism was similar between hypertensive subjects and controls from a Brazilian birth cohort (Fernandes-Rosa et al., 2009).

MRc.-2G>C (rs2070951) is a frequent SNP located in the 5'-untranslated region of the NR3C2 gene, two nucleotides upstream of the first translation start site. The C allele of MRc.-2G>C has been associated with lower basal cortisol levels (Kuningas et al., 2007) and a decrease in MR-dependent transcriptional activation in the presence of aldosterone (Arai et al., 2003). However MRc.-2G>C has not been tested for associations with salt handling and the precise mechanism of action of this SNP is currently unclear.

In this study, we first investigated the functionality of MRc.-2G>C by testing its effect on MR protein expression, its influence on the MR-A/MR-B protein ratio and the transactivational activity *in vitro* with different ligands. We then assessed the effect of this polymorphism on sodium handling and regulation of the renin-angiotensin-system in normotensive healthy subjects included in a crossover study to receive a low Na-high K or a high Na-low K diet and in a group of mildly

hypertensive patients exposed to a salt sensitivity (Weinberger's) test. Finally, we tested for an association between BP measures in a large multi-site cohort for depression and anxiety.

Materials and Methods

Construction of the plasmids

The recombinant pRSV human MR (hMR) plasmid, containing the last 30 base pairs of exon 1α to the untranslated region of exon 9, was obtained from Dr. R. Evans (gene expression laboratory and HHMI, The Salk Institute for Biological Studies, La Jolla, Ca). The MR c.-2G>C site was mutated from G to C with the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the primers 5'-GGCCGAGGCAGCGATGGAGACCAAAG-3' and 5'-GCTGCCTCGGCCCTTTGGTCTCCAT-3' according to the manufacturers protocol (Fig. 1c). The plasmids expressing only MR-A or MR-B were generated by mutating the second or the first ATG into ATC by using the primers 5'TGAAGGTCTAGATACGGAAAGACGGTGG-3' and 5'CCACCGTCTTTCCGTATCTAGACCTTCAG-3' or 5'CCGAGGCAGGGACGGAGACCAAAGG-3' and 5'CCTTTGGTCTCCGTCCCTGCCTCGG-3' respectively. After mutagenesis the hMR insert of the plasmid was sequenced to assure absence of other mutations. Plasmids were purified from DH5α E-coli bacterial cultures using the Pure Yield purification system (Promega, Leiden, The Netherlands).

To generate pcDNA3_1α2G-luc and pcDNA3_1β2G-luc (Fig. 1a and b), human kidney and hippocampus (for pcDNA3_1α2G-luc) cDNA was amplified to generate fragments containing exon 1α or 1β and 60 bp of exon 2 of the NR3C2 gene. The cDNA coding for luciferase was amplified, starting from codon 2, using the pGL2-basic vector (Promega, Madison, WI) as a template. Primers were designed containing specific restriction sites for subsequent cloning. All amplification fragments were subcloned into pGEMTeasy (Promega). The intermediate constructs pGEMTeasy_1α2 and pGEMTeasy_1β2 were digested with SpeI/XbaI, and ligated to the luciferase cDNA previously excised from pGEMTeasy_luc by digestion with SpeI. The chimeric construct 1α2luc was excised from pGEMTeasy_1α2luc by digestion with BamHI and XhoI and inserted into pcDNA3 (Invitrogen, Paisley, Scotland) to obtain pcDNA3_1α2-luc and pcDNA3_1β2-luc. Oligonucleotides used for amplification of the different fragments are the following:
5'-ATGGATCCAGAGGAAGCCCGTGCAGTCA 3' and 5'-CCCACCGTCTTTCCATATCT-3'
1β2 : 5'-ATGGATCCCGCCGCTGCCTCGCCGCCTC-3' and 5'-CCCACCGTCTTTCCATATCT-3'
Luciferase: 5'-GCACTAGTCGAAGACGCCAAAAACATAAAGA-3' and 5'-CCCTCGAGCATTTTACAATTTGGACTTTC-3'
Different pcDNA3_1α2-luc and pcDNA3_1β2-luc clones were sequenced to verify the nucleotide rs2070951 (MR c.-2G>C). The G to C change was created by site-directed mutagenesis using the Quick change site-directed mutagenesis kit (Strategene, La Jolla, CA) on the recombinant plasmids. The following sense primer was used together with their corresponding antisense oligonucleotide.

Transactivation assays

Rabbit RCSV3 cells derived from kidney cortical collecting duct (Vandewalle et al., 1989) (kindly provided by Pr. P. Ronco, Hôpital Tenon, Paris) were grown as described previously (Zennaro et al., 2001) and transfected using lipofectamine 2000 (Invitrogen) with 0.25 µg of plasmid containing either MR-2G or MR-2C pcDNA3, 0.625 µg of a GRE2_TATA_luc reporter plasmid plasmid (Asselin-Labat et al., 2004) and 0.25 µg of pSVβgal. The day after transfection, steroids were added at different concentrations and 48 hours after transfection, luciferase and β-galactosidase activities were assayed using the Dual-Light® System and the Galacton-Plus® Substrate (Applied Biosystems). Results were standardized for transfection efficiency and expressed as the ratio of luciferase activity over β-galactosidase activity in arbitrary units.

Cos-1 cells (African green monkey kidney cells) were cultured as described previously(van Leeuwen et al., 2010) and seeded in 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 3 x 10^4 cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using SuperFect (Qiagen, Venlo, The Netherlands). Plasmids containing MR-2G or MR-2C or no MR (control) and the reporter plasmid TAT3-Luc (tyrosine amino transferase triple hormone response element) were used at 200 ng/well. Construction of the plasmids is described in the data supplement available online. The control plasmid pCMV-Renilla (Promega, Leiden, The Netherlands) was used at 2 ng/well. To exclude variation due to impurity or concentration of the plasmid each plasmid was cultured 3 times, purified and tested. In each of the three experiments the plasmids were tested in quadruplicate. One day after transfection, the cells were treated with different concentrations of either aldosterone or cortisol (both Sigma-Aldrich, Zwijndrecht, the Netherlands). After 24h of incubation the cells were harvested in passive lyses buffer (Promega) and firefly and Renilla luciferase activity was determined using the dual label reporter assay (Promega) and a luminometer (CentroXS, Berthold, Bad Wildbad, Germany).

Protein expression studies

For studies investigating the effect of the c.-2G>C SNP on protein synthesis, rabbit RCSV3 cells and COS-7 cells were seeded in six-well plates at a density of 3x10^5 cells per well at least 6 hours before transfection in fresh medium without any added steroid. Cells were transfected by the calcium phosphate method with 0.66 µg of plasmids pcDNA3_1 β 2G-luc, pcDNA3_1 β 2C-luc, pcDNA3_1α2G-luc or pcDNA3_1 α 2C-luc. Co-transfection of 0.16 µg pSV β gal (Clontech, Palo Alto, CA) was performed to normalize for transfection efficiencies. Cells were rinsed twice with cold PBS, lysed in lysis buffer and cellular extracts assayed for luciferase and β -galactosidase activities as previously described(Zennaro et al., 2001). Results were expressed as the ratio of luciferase activity over β -galactosidase activity in arbitrary units.

For Western blot Cos-1 cells were seeded in 6-well plates (Greiner Bio-One) at 2 x 10^5 cells/well. The cells were transfected the next day using Trans-it Cos transfection reagent (Mirus, Madison, USA). Plasmids containing one of the hMR variants, e.g. hMR-2G, hMR-2C, mutated hMR only expressing MR-A and mutated hMR only expressing hMR-B or no hMR (control) were used at 2µg/well. Cells were harvested 48 hours after transfection. The Western blots using primary antibodies MR 1D5, detecting amino acid 1-18 and therefore only MR-A, and 2B7, detecting amino

acid 64-82 and therefore both MR-A and MR-B, (a generous gift by Gomez-Sanchez, Division of endocrinology, University of Mississippi, Jackson, MS) were performed as described previously(Conway-Campbell et al., 2007) The differences in intensity of the MR bands were quantified with Image J (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/)

RNA isolation and real time quantitative PCR

All reagents used were from Invitrogen unless otherwise specified. Transfections with plasmids containing one of the hMR variants, pcDNA3_1α2G-luc, pcDNA3_1β2C-luc, pcDNA3_1β2G-luc, pcDNA3_1β2C-luc and pSVgal were performed as described under the protein expression section. For the hMR transfections 24 hours and for other transfections both 12 and 24 hours after transfection, total RNA was extracted in Trizol reagent according to manufacturer's recommendations. Total RNA was treated with DNase I and quantified with the Ribogreen RNA quantitation kit as previously described (Zennaro et al., 2001). For the transfections with hMR 1μg and for the others 500 ng of RNA was used to generate cDNA. Amplification of the cDNA was performed using SYBRgreen (qPCR MasterMix Plus for SYBR® green I, Eurogentec, Seraing, Belgium) on a Chromo4 Continuous Fluorescence Detector (MJ Research, Bio-Rad laboratories, Waltham, MA), according to the manufacturers instructions. Primer sequences are available upon request. Controls without reverse transcriptase and without template were included to verify that fluorescence was not overestimated by residual genomic DNA amplification or from primer dimer formation. Moreover RT-PCR products were analyzed in a post-amplification fusion curve to ensure that a single amplicon was obtained. Ribosomal 18S RNA was used to normalize for RNA quality, quantity and RT-efficiency. Quantification of β gal was used to normalize for transfection efficiency. Quantification was done by the standard curve method. Standard curves were generated by serial dilutions of a linearized plasmid containing the specific amplicon, spanning six orders of magnitude, yielding a correlation coefficient of at least 0.98 in all experiments. For all experiments, PCR efficiency was close to 2 indicating a doubling of DNA at each PCR cycle, as expected.

Subjects

In all studies respondents provided written informed consent and all studies were performed in accordance with the Declaration of Helsinki guidelines.

Mild hypertensive group. Ninety Italian patients (34 females, 56 males, mean age 46.0 yrs, mean body mass index 26.8) with mild essential hypertension , i.e. mean systolic blood pressure (SBP) 152 mm Hg and mean diastolic blood pressure (DBP) 97.5 mm Hg were recruited by 9 medical centres. The patients were taken off antihypertensive medication 14 days prior to testing. After a normal sodium diet (150 mmol/day) for 3 days, patients were subjected to an acute salt-loading (constant rate intravenous infusion of 2 L of 0.9% NaCl carried out over 4 hours) and salt-depletion protocol (sodium restriction 50 mmol plus three doses of 37.5 mg of furosemide) to evaluate the distribution of BP sensitivity to salt (Strazzullo et al., 2000a). If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period was greater than the median (10 mmHg), the patient was classified as "salt-sensitive" otherwise the patient was considered "salt-resistant". Twenty-four hours urinary sodium excretion, upright plasma aldosterone (after two hours orthostatism) and plasma renin activity were measured after three days of normal sodium diet (150

mmol/day) just before the salt-load. Post-load plasma aldosterone and renin activity were measured 4 h after the beginning of the salt-load. Urine electrolytes analyses, measurements of plasma renin activity and plasma aldosterone concentration were performed as previously described (Strazzullo et al., 2000b).

Healthy group

Forty healthy French Caucasian normotensive (BP less than 140/90 mmHg in the supine position after 5 minutes of rest) males (18–35 years) were included in a crossover study to receive both a low Na+ (less than 20 mmol NaCl/day) and high K+ (more than 140 mmol Kcl/day; low Na+–high K+ diet) or high Na+ (more than 250 mmol NaCl/day) and low K+ (less than 50 mmol Kcl/day; high Na+–low K+ diet) for 1 week. The study design has been previously described in detail (Azizi et al., 2005) and all subjects completed the study after giving written informed consent. Procedures were in accordance with institutional guidelines. Controlled Na+/K+ diet periods were separated by a 7-day washout period. On the ad libitum Na+ and K+ diet at baseline and on day 7 of each controlled Na+/K+ diet period, blood was sampled at 0900 in the fasting state after 1 hour of rest in the sitting position for plasma immunoreactive active and total renin and plasma aldosterone and ANP determinations. Urine was collected in two 12-hour periods from 0800 to 2000 and from 2000 to 0800 and was used for hormone and electrolyte determinations. The methods used for collecting blood samples and for quantifying plasma active renin, total renin, atrial natriuretic peptide (ANP) and aldosterone were as described previously (Azizi et al., 2005).

Multi-site cohort for depression and anxiety

Data were obtained from the Netherlands Study of Depression and Anxiety (NESDA), an 8-year longitudinal cohort study that includes 2,981 Dutch Caucasian participants, aged 18 through 65 years, A detailed description of the study design and sample has been published previously (Penninx et al., 2008). NESDA is a multi-site cohort study to describe the long-term course and consequences of depressive and anxiety disorders in which cardiologic parameter such as blood pressure were analysed (Penninx et al., 2008). Participants were recruited from different locations in the Netherlands (Amsterdam, Leiden and Groningen). For the current study, data were used from the baseline interviews conducted between September 2004 and February 2007. Of the 2981 participants, 1860 subjects have been genotyped and after quality control (previously described (Sullivan et al., 2009)), 1754 subjects (67.9% woman, mean age 42.35, SD 12.49 years) were included in the study. The cohort consisted predominantly of subjects with current or remitted anxiety and/or depressive disorders at time of BP assessment. However, the presence of psychiatric diagnosis was not associated with BP. BP was registered by the OMRON IntelliSense Professional Digital Blood Pressure Monitor, HEM-907XL (Omron Healthcare, Inc). SBP and DBP were measured twice during supine rest on the right arm and were averaged over the 2 measurements. A correction was made for all of the individuals on hypertensive medication, which was considered as being used if subjects frequently (50% of days in last month) used antihypertensives (ATC code C02), diuretics (ATC code C03), β-blocking agents (ATC code C07), or calcium channel blockers (ATC code C08). In accordance with earlier studies and based on the efficacy of antihypertensive drugs in randomized trials (Mancia and Parati, 2004; Cui et al., 2003) we added 10 mm Hg to SBP and 5 mm Hg to DBP for subjects who used antihypertensives.

Determination of genotypes

Patients from the Italian cohort were genotyped by direct sequencing after PCR amplification using an ABI BigDye termination sequence kit v1.1 (Applied Biosystems Foster City, USA) on an ABI 3700 DNA analyzer and the reaction was performed according to the manufacturer's specifications. Patients from the French cohort were genotyped by direct sequencing of PCR products using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3700 DNA Analyzer. Primers for PCR amplification are Primers used for genotyping are:

Fw 5'-ATA TGT TTT GTG GCT TAG CAA AT-3'

Rv 5'AAC TTA GAG TGG AAG GAC GAT GG-3"

Subjects from the Dutch NESDA cohort were genotyped by Perlegen Sciences (Mountain View, CA, USA) using a set of four proprietary, high-density oligonucleotide array , which had a call rate of 98.9% for this SNP. A detailed description of the genotype process is described elsewhere (Sullivan et al., 2009).

Statistical analysis

In cell culture experiments the difference between the two alleles was analyzed with a two-way ANOVA with Bonferroni post-hoc tests. For western blot the difference in protein expression was analyzed with t-test. In vitro results are shown as the mean ± SD or mean ± SEM. Statistical analysis of all the *in vitro* results was performed with Graph Pad Prism version 5 (GraphPad software Inc, San Diego, CA).

In the Italian mild hypertensive group, the MR genotype effects were assessed using two-way (genotype/sex) ANCOVA with age and BMI as covariates. Skewed variables were log-transformed before statistical testing. When the interaction between genotype and sex was significant (blood pressure), analyses were separately performed in each sex. The statistical analysis was performed using Systat 11 statistical software.

In the healthy volunteers group, differences between MR genotype groups were assessed by ANOVA for a crossover design for plasma parameters and by non parametric Kruskal-Wallis test for urinary parameters. Stata Statistical Software (version 7.0; StataCorp.) was used for statistical analysis.

In the Dutch NESDA cohort, differences between MR genotype groups in SBP and DBP were analysed using general linear model (GLM). We tested for gene gender interaction and performed the GLM for the entire sample and split for gender. The previous identified confounders related to BP in this cohort: gender, age, years of education, alcohol use, smoking, tricyclic antidepressants, noradrenergic and serotonergic working antidepressants, number of chronic disease, body mass index, depression and anxiety comorbidity and presence of significant life events (Licht et al., 2009) were included as covariates. SPSS 15.0 was used for the statistical analysis. Association study data are expressed as means ± SD or medians and interquartile ranges.

Allele frequencies in the three cohorts were calculated and analyzed for deviation from Hardy-Weinberg equilibrium (HWE) using Haploview. The groups consisting of mild hypertensive individuals and of healthy individuals did not deviate from HWE, as well as the healthy individuals from the NESDA cohort. However, deviation from HWE was observed in the patients with mood and/or anxiety disorders from the NESDA cohort ($p=0.0053$). Deviation from HWE in patients can

be interpreted as an indication of association of particular genes with disease (Lee, 2003; Deng et al., 2000). Under these circumstances, the lack of HWE arises as a result of selection according to the phenotype that results in allele and genotype distributions that are nonrandom.
Data are expressed as mean ± SD or otherwise specified. A P-value of less than 0.05 was considered to be significant.

Results

MRc.-2G>C influences the transactivation activity of the MR.
The influence of the MR-2G>C SNP on MR function was measured with an *in vitro* transactivation assay. Cos-1 cells and RCSV3 cells were transfected with plasmids containing either MR with the -2 C nucleotide or MR with the -2 G nucleotide (Fig. 1c).

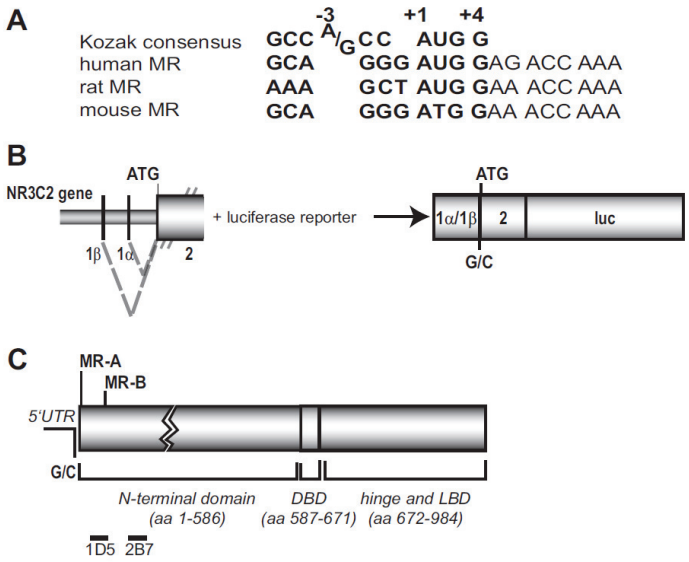

Figure 1. Presentation of the sequence context surrounding the SNP c.-2G>C, schematic representation of the chimeric gene constructs and schematic representation of hMR isoforms MR-A and MR-B . a) Alignment of the Kozak consensus sequence for translation initiation and the sequence upstream of the principal AUG codon (pos. +1) of the MR mRNA. The SNP MRc.-2G>C is located at position -2 with respect to the AUG. The human MR mRNA sequence is aligned with the rat and mouse MR mRNAs. b) Schematic representation of the chimeric constructs 1α2luc and 1β2luc (c) schematic representation of hMR isoforms MR-A and MR-B and the location of the antibodies used in westenblot for detection of the two isoforms.

After incubation with aldosterone (concentration range 0 to 10^{-7}M) or cortisol (concentration range 0 to 10^{-6} M) luciferase activity was measured. The -2C allele showed significantly higher transcriptional activity than the -2G variant using either aldosterone or cortisol in both cell models (Cos-1 cells: $p<0.05$, $p<0.001$ S12a and S1b; RCSV3 cells: $p<0.001$ and $p<0.001$ for aldosterone and cortisol respectively, (Fig. 2).

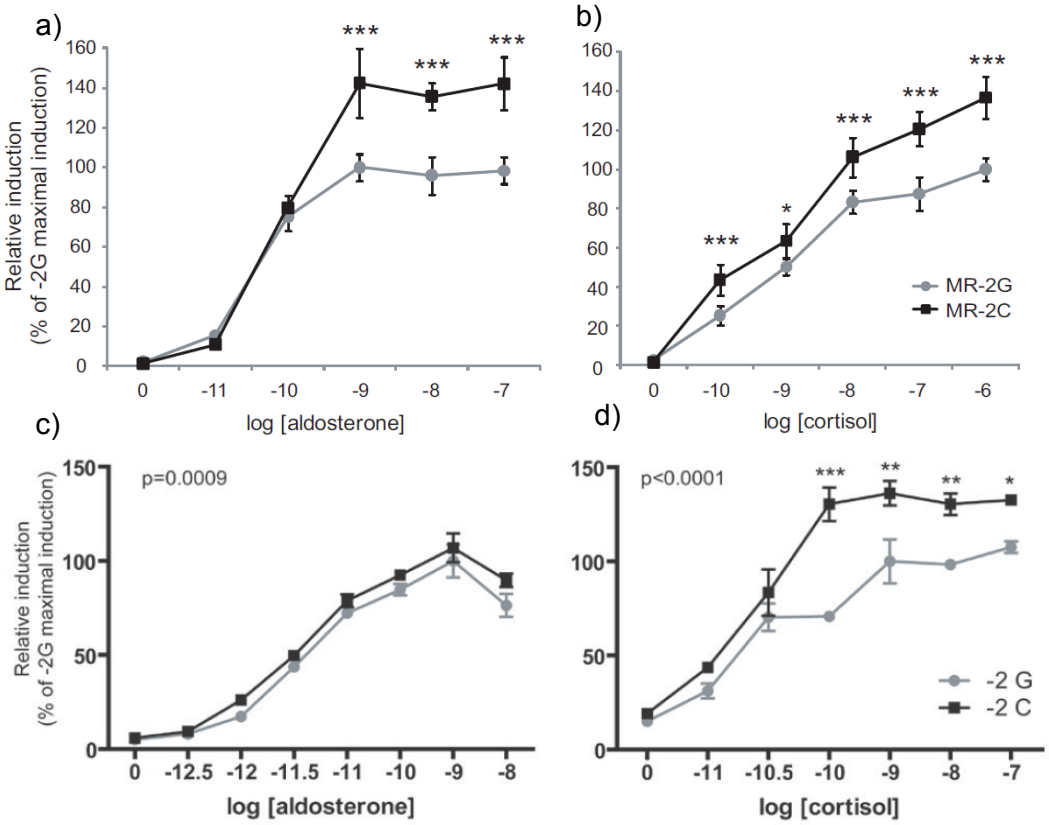

Figure 2. Aldosterone and cortisol-driven transactivation by MR c.-2C and MRc.-2G. Aldosterone (2a and 2c) and cortisol (2b and 2d) driven transactivation capacity of the MRc.-2G>C variants on a TAT-3 promoter in Cos-1 cells (2a and 2b) and RCSV3 cells (2c and 2d) displayed as percentage of maximal induction obtained with -2G (±SEM, c) and d)). The MR-2G variant showed a lower transactivation compared with the MR-2C variant. Results represent at least two independent experiments performed in triplicate.

MRc.-2G>C affects protein expression independently of the 5'untranslated region.

In the human NR3C2 gene, two 5'-untranslated (UT) exons are alternatively transcribed and generate two different mRNAs which code for a unique MR protein, since the translational start site is located at the beginning of exon 2. The c.-2G>C SNP is located 2 nucleotides upstream of the translation initiation site in the middle of the Kozak consensus sequence for translational initiation (Kozak, 1986) (Peri and Pandey, 2001) which is highly conserved among the NR3C2 genes from several species (Fig. 1a). In order to test a possible functional role of the c.-2G>C SNP, we have investigated its influence on translational efficiency, in the context of both 5'-UT exons 1α and 1 β. Using chimeric constructs, with exon 1 α or 1β inserted together with the Kozak sequence containing either G or C immediately upstream of the coding sequence of the luciferase gene (Fig. 1b), transient transfection assays were performed in renal RCSV3 cells and in COS cells, the amount of luciferase activity representing the amount of protein generated. In the presence of comparable mRNA levels 12 hs post-transfection, the C allele was associated with significantly higher protein levels compared to the G allele, both at 12 and 24h post-transfection in RCSV3 cells ($p<0.0001$, Fig. 3a) and Cos-7 cells ($p<0.0001$, Fig. 3b). This effect was observed in the presence of both the UT exons 1α or 1β. Interestingly, luciferase activity in the presence of the UT region 1 β was ~ 40% of that observed with exon 1 α ($p<0.0001$).

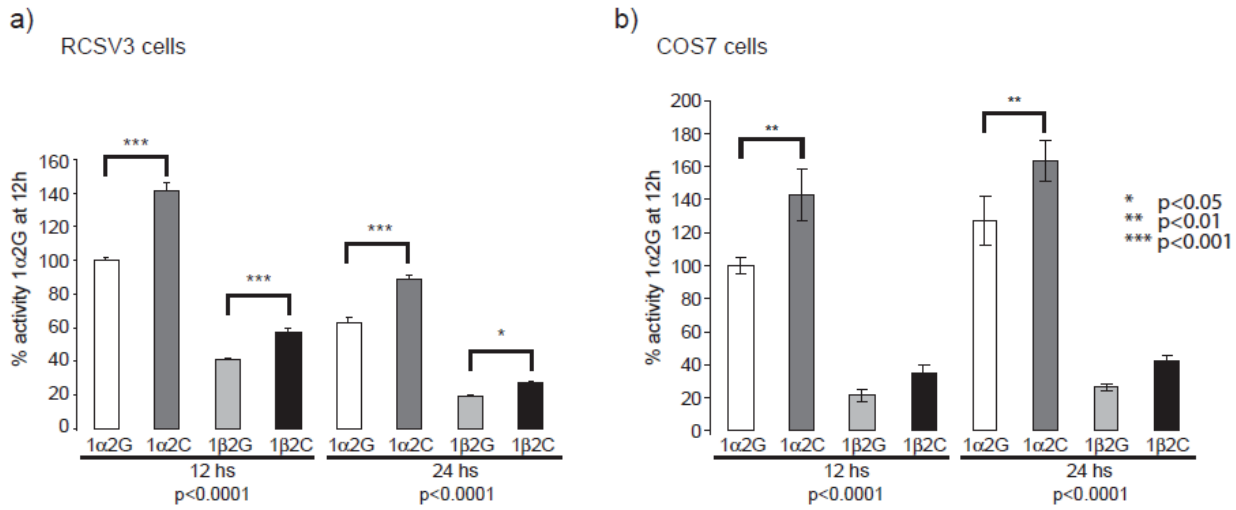

Figure 3. Effects of MRc.-2G>C on protein expression**.** Effects of -2G>C variants on protein expression were measured with a recombinant MR-luciferase construct. Chimeric constructs containing exon 1α (white and dark gray bars) showed a higher luciferase expression compared to chimeric constructs containg exon 1β (light gray and black bars) in either RCSV3 cells (3a) or COS-7 cells (3b). The MR-2C variant (dark gray and black bars) showed a higher luciferase expression compared with the MR-2G variant (white and light gray bars) in either RCSV3 cells (3a) or COS-7 cells (3b).

We then investigated the hypothesis that the -2G>C SNP may affect alternative translation of the previously described human MR isoforms MR-A and MR-B. Constructs containing either MR with the -2 C nucleotide or MR with the -2 G nucleotide and control constructs expressing only MR-A or MR-B were transiently transfected in Cos-1 cells. The MR mRNA levels after transfection were similar for MR -2C and MR-2G (Figure 4).

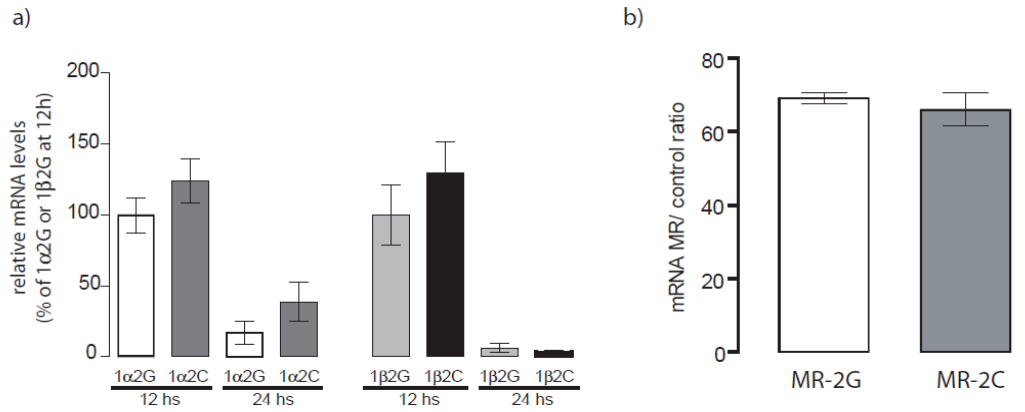

Figure 4. Effects of MRc.-2G>C on the expression of MR isoforms. Constructs containing MR with either -2C or -2G and control constructs expressing only MR-A or MR-B were transiently transfected in Cos-1 cells. The effect of the -2G or -2C allele on MR-A and B protein expression was measured by Western blot using two different antibodies recognizing aminoacids 1 to 18 (visualizing MR-A, Fig. S3a), or aminoacids 64 to 82, detecting both MR-A and B (Fig. S2b). c) Quantification of MR expression of multiple experiments using the MR-A detecting antibody (1D5).

Two different primary antibodies were used in Western blots: 1D5 directed against aminoacids 1 to 18 was used to visualize MR-A, while 2B7 directed against aminoacids 64 to 82 was used to detect MR-A and B (fig. 1c). Specificity of the antibodies was confirmed with the control constructs expressing only MR-A or only MR-B In Cos-1 cells transfected with the constructs MR -2G or MR

-2C only MR-A was expressed but not the MR-B. Western blots also confirmed the differences in MR-A protein expression between -2G and -2C containing constructs, the MR-2C resulted in significantly increased MR-A protein expression, the ratio between MR and the control tubulin was 0.300 (±0.014) for MR-2G and 0.362 (±0.022) for MR-2C (Fig. 5)

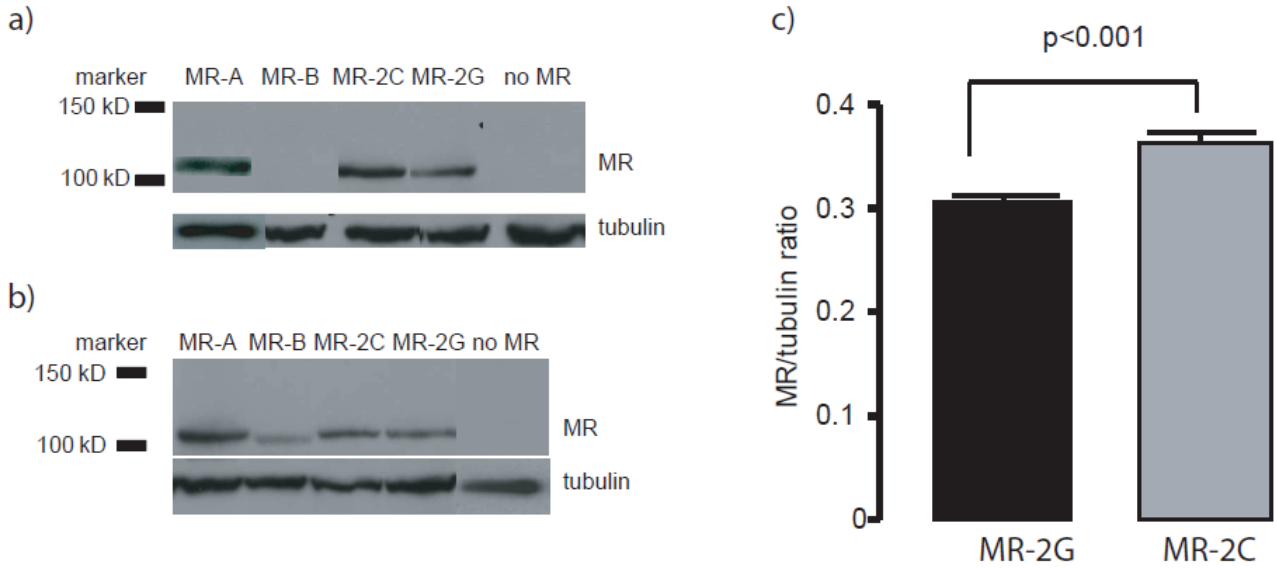

Figure 5. Effects of MRc.-2G>C on the expression of MR isoforms. Effect of -2G>C variants on MR-A and B protein expression was measured by Western bot using antibodies directed against a) the first 18 aminoacids of the MR, therefore only detecting MR-A and b) aminoacid 64-82 detecting MR-A and MR-B. c) Quantification of MR expression.

Consequences of MRc.-2G>C on renal sodium handling and blood pressure regulation

The MRc.-2G>C SNP was determined in subjects from the three independent cohorts. Allele and genotype frequencies were not significantly different between the three groups (χ^2_2= 5.36, p=0.07 and χ^2_4=6.57, p=0.16, resp.) (Table 1).

Table 1 Comparison of allele and genotype frequencies in the three different cohorts

group	Allele frequencies		Genotype frequencies		
	C	G	CC	GC	GG
Hypertensive	0.59	0.41	0.32	0.53	0.15
Healthy	0.51	0.49	0.25	0.53	0.22
NESDA	0.51	0.49	0.24	0.53	0.23

Mild hypertensive group. After 3 days of high salt diet (150 mmol/day), plasma renin activity was significantly higher in mild hypertensive GG patients compared to the other genotypes (Table 2). In addition, the GG genotype was associated with significantly higher SBP levels in men, but not in women (Table 3). No association of the MRc.-2G>C genotypes was found with other parameters tested in the cohort, such as 24-hours urinary sodium excretion and plasma aldosterone or plasma aldosterone and plasma renin activity levels after salt loading. Following the Weinberger's test, the ratio of salt sensitive to salt resistant subjects was not significantly different among genotypes (χ^2_1=1.69, P=0.43) and there was no relationship between the genotypes and blood pressure response to the salt sensitivity test (p=0.21, data not shown).

Table 2. Characteristics (mean ± SD) or geometric mean (95%CI) in 90 mild hypertensive men and women from the Italian cohort according to the MR MR-2G/C genotype

Italian cohort		3 days controlled salt intake (150 mmol/day)			Weinberger's test	
90 subjects	Freq (%)	24hr Urinary sodium excretion/creatine (mEq)	Aldosterone (ng/dl)	Plasma renin activity (ng/ml/h)	Salt Resistant (ΔBP< 10mm Hg) n	Salt Sensitive (ΔBP >10 mm Hg) n
MR c.-2CC (n=29)	32.2	155.1 ± 54.3 (n=25)	12.8 (10.1-16.2) (n=28)	1.42 (1.07-1.88) (n=28)	17	12
MR c.-2GC (n=48)	53.3	172.7 ± 83.7 (n=39)	12.8 (10.6-15.4) (n=47)	1.46 (1.20-1.79) (n=47)	21	27
MR c.-2GG (n=13)	14.8	180.1 ± 50.6 (n=11)	12.9 (9.5-17.6) (n=11)	2.25 (1.33-3.79) (n=11)	7	6
P-value:		0.69*	0.65*	0.029†	0.43‡	

* genotype effect from 2-way (genotype, sex) ANCOVA adjusted for age, BMI. There was no interaction between sex and genotype for any variable. For aldosterone and renin, log values were used in the statistical tests. †Global test (2df): 0.10, GG vs CC+GC. ‡$\chi^2 = 1.69$, df = 2

Table 3. Association analysis of the MR MR-2G/C genotype with diastolic and systolic blood pressure in 90 mild hypertensive men and women from the Italian cohort

Blood pressure	Group	MR-2G/C	Mean	SD	P*
Systolic (mmHg)	Men (n=56)	CC (22)	151.5	11.6	0.004
		GC (26)	147.8	12.6	
		GG (8)	165.8	9.8	
	Women n=34	CC (7)	151.0	9.8	0.21
		GC (22)	158.1	18.9	
		GG (5)	141.0	8.9	
Diastolic (mmHg)	Men (n=56)	CC (22)	99.1	5.1	0.053
		GC (26)	96.5	6.3	
		GG (8)	102.0	5.3	
	Women n=34	CC (7)	95.6	8.3	0.056
		GC (22)	98.1	9.7	
		GG (5)	90.0	3.5	

BP: blood pressure; *genotype effect by ANCOVA adjusted for age and BMI in each sex separately. Interaction genotype X sex: P = 0.001 and P = 0.005 for PAS and PAD respectively.

Normotensive subjects. On the *ad libitum* Na^+/K^+ diet, 156 mmol of Na^+ (interquartile range, 126–187 mmol) and 68 mmol of K^+ (interquartile range, 59–81 mmol) were excreted in the urine in 24 hours. By controlling the Na^+/K^+ intakes, it was possible to achieve the desired Na^+ and K^+ balances, as reflected by 24-hour urinary NaCl and KCl excretion rates (Table 4). The 24-hour

urinary Na^+ and K^+ excretion levels were identical for subjects of the 3 genotypes for all of the diets (Table 4).

Plasma active and total renin and aldosterone, and ANP on the *ad libitum* Na^+/K^+ diet were within the physiological ranges and did not differ according to genotype (Table 4). As expected, plasma active and total renin and aldosterone concentrations increased with the low Na^+–high K^+ diet and decreased with the high Na^+–low K^+ diet. The changes in plasma ANP concentrations were in the opposite directions (Table 4). On the high Na^+ – low K^+ diet, GG subjects had significantly higher levels of plasma active and total renin and aldosterone concentrations than CC subjects, with heterozygous GC subjects presenting intermediate values. A similar trend was observed on the low Na^+–high K^+ diet, but differences between GG and CC genotypes were not significant.

Table 4. Biological characteristics of normal volunteers according to the MR MRc.-2G>C genotype at baseline and after 7 days of High Na-Low K and Low Na-High K diet.

	Baseline	High Na+ - Low K+	p	Low Na+ - High K+
Plasma active renin (pg/ml)				
CC (n=10)	13.8 [9.7;19.7]	5.8 [4.3;7.8]	*	30.7 [24;39.2]
GC (n=21)	14.3 [11.6;17.6]	8.4 [6.5;11]		38.5 [32.6;45.4]
GG (n=9)	15.8 [11.7;21.4]	10.9 [6.7;17.7]		43.6 [30;63.3]
Plasma total renin (pg/ml)				
CC (n=10)	108.3 [83.2;141.1]	62.4 [47.4;82]	*	142.3 [116;174.5]
GC (n=21)	125.4 [106.6;147.5]	91.7 [73.8;113.9]		169.3 [141.8;202.1]
GG (n=9)	112.5 [94.9;133.4]	83.2 [62.9;110]		156.9 [119.3;206.5]
Plasma ANP (pg/ml)				
CC (n=10)	23.3 ± 4.6	26.7 ± 8.9		17.9 ± 4.2
GC (n=21)	20.6 ± 4.6	25 ± 10.4		16.4 ± 3.3
GG (n=9)	20.3 ± 5.5	22.6 ± 8.4		16.4 ± 3.8
Plasma aldosterone (pg/ml)				
CC (n=10)	63.7 [47.7;85.1]	25.7 [18.3;36.1]	*	295.1 [216.4;402.5]
GC (n=21)	73 [64.2;83.1]	38.6 [31.6;47.2]		319.9 [262;390.4]
GG (n=9)	69.3 [52.8;90.8]	38.5 [30.7;48.3]		338.9 [266.1;431.5]
24 h urine volume (mL)				
CC (n=10)	1663 (1227;1980)	2092 (1896;3110)		2415 (1777;3463)
GC (n=21)	1496 (1187;1943)	1910 (1540;2610)		2123 (1781;2711)
GG (n=9)	1563 (1188;2286)	2448 (1918;2662)		2401 (2259;2976)
24h urinary sodium excretion (mmol/24h)				
CC (n=10)	177 (153.6;237.5)	277.4 (253.5;300.4)		17.2 (13.9;20)
GC (n=21)	138.9 (108;167.1)	246.2 (218.8;278.5)		14.1 (11.7;21.9)
GG (n=9)	158.2 (133;188)	242.9 (230;298)		17.4 (11.3;30.6)
24h urinary potassium excretion (mmol/24h)				
CC (n=10)	72.2 (65.8;86.6)	36.9 (26;47.2)		132.1 (96.5;144.9)
GC (n=21)	68.8 (55.3;83.1)	39.6 (34.3;43.1)		119.9 (106.1;140.8)
GG (n=9)	67.4 (57.6;72.2)	39.3 (29.9;42.6)		111.2 (94.5;132)

* : $p<0.05$ vs GC and vs GG. Data are mean [95% CI], mean ± SD or median (interquartile range)

NESDA cohort. There was a significant association between SBP and MRc.-2G>C (p= 0.041), even after adjustment for counfounding factors (Table 5). GG subjects had significant higher systolic blood pressure than GC or CC subjects (mean SBP for GG: 138.2±1.9 mmHg, GC: 137.3±1.8 mmHg and CC: 135.2±1.9 mmHg). Although we did not detect a gender x genotype interaction (p=0.36), we performed a separate analysis for both men and women. The association with MRc.-2G>C and SBP was significant for men but not for women (Table 5). Men with the GG genotype had significant higher systolic pressure (5.17 mm Hg) than those with the CC genotype (p= 0.15). SBP among men was 147.0±21.2 mmHg for GG genotype, 143.8±17.6 mmHg for GC genotype and 141.9 ± 20.6 mmHg for CC genotype.

Table 5. Association analysis of the MR MR c-2G>C genotype with diastolic and systolic blood pressure in Dutch cohort

Blood pressure	Group	MR-2G/C	Mean	SD	P*
Systolic (mmHg)	Men (n=563)	CC (137)	141.86	20.64	0.046
		GC (304)	143.81	17.57	
		GG (122)	147.03	21.23	
	Women (n=1191)	CC (262)	129.53	22.04	0.195
		GC (634)	131.26	18.96	
		GG (295)	131.03	17.76	
Diastolic (mmHg)	Men (n=563)	CC (137)	83.38	12.24	0.232
		GC (304)	84.83	11.07	
		GG (122)	85.49	12.71	
	Women (n=1191)	CC (262)	79.29	13.17	0.301
		GC (634)	80.01	11.41	
		GG (295)	80.26	11.08	

BP: blood pressure; *genotype effect by GLM adjusted for age, presence of life events, years of education, smoking, alcohol abuse/dependence, use of tricycle antidepressant (TCA), use of noradrenergic serotonergic (NS) antidepressant, number of chronic disease and BMI in each gender separately.

Table 6. Results of General Linear Model with mean systolic pressure (medication adjusted) as outcome in the whole cohort (n=1754 subjects), and stratified for gender

Parameter	complete cohort			Gender					
				Male (n=563)			Female (n=1191)		
	F test	P	R^2	F test	P	R^2	F test	P	R^2
Model			0.263			0.145			0.207
MR c.-2>C	3.202	0.041		3.088	0.046		1.639	0.195	
Negative life events,	1.104	0.294		0.327	0.568		0.895	0.344	
Gender	141.204	<0.001							
Age	246.499	<0.001		46.926	<0.001		198.771	<0.001	
Years of education	5.799	0.016		0.398	0.528		5.556	0.019	
Smoking	9.603	0.002		8.915	0.003		2.966	0.085	
Alcohol	0.728	0.394		0.187	0.665		0.417	0.519	
Use of a TCA	4.241	0.040		1.439	0.231		2.638	0.105	
NS antidepressant,	2.711	0.100		0.333	0.564		2.739	0.098	
chronic diseases	11.376	0.001		5.885	0.016		6.001	0.014	
BMI	9.427	0.002		4.747	0.030		5.584	0.018	

Abbreviations: TCA, tricycle antidepressant; NS, noradrenergic serotonergic; BMI, Body Mass Index

Discussion

In this study we have undertaken the functional analysis of the c.-2G>C polymorphism, a frequent SNP in the NR3C2 gene coding for the MR, and its selective genotyping in subjects from different cohorts. The c.-2G>C variant is associated with differential expression of the MR *in vitro*; importantly, *in vivo*, this SNP influences circulating levels of plasma aldosterone and renin.

A possible role of the MRc.-2G>C polymorphism on translational efficiency had been suggested based on its location 2 nucleotides upstream of the first translation initiation site in the middle of the Kozak consensus sequence for translational initiation (Kozak, 1986). First, we showed with two different endogenous ligands and in two different cell lines that MR translated from a construct carrying a C at position -2 was associated with a higher transcriptional response *in vitro*. These results are in contrast to previous work describing lower transactivation of a reporter gene by the -2C allele using aldosterone (Arai et al., 2003). Differences in methodology or the cell line that was used might explain this result. Second, using three different approaches and two different cell lines, we showed that the C allele results in more abundant protein expression than the G allele. Since mRNA expression and the affinity of the MR are not modified by MRc.-2G>C it is concluded that this polymorphism influences translation. This is in accordance with data showing that a C at position -2 is probably more efficient for translation, since in 1534 human transcripts the sequence surrounding the initiation codon contains a C at position -2 in 40% of cases, while a G nucleotide is present in only 18% of transcripts (Peri and Pandey, 2001). Third, transcription of two different exons from alternative promoters on the NR3C2 gene can lead to different transcripts, with two different 5'-UT regions that can precede MRc.-2G>C. Our results demonstrate that the 5'UT has no influence on the observed effect of the SNP. With both exons 1α and 1β the -2C allele was associated with higher protein translation. However, protein expression in the presence of the UT

region 1 β was ~ 40% of that observed with exon 1α, indicating that the sequence of exon 1 β is less optimal for translation. Finally, the influence of MR -2G>C on the MR-A/MR-B ratio was investigated. In Western blot experiments the MR-A isoform, translated from the first translation start, was more abundant in the presence of the C allele. Transient transfection of MR containing either the -2 C or -2 G allele did not result in any detectable MR-B protein. As far as we know, only one study reported the MR-B isoform (Pascual-Le Tallec et al., 2004); in that study MR-B was detected with an *in vitro* translation assay. Our results suggest that MR-B is not translated, at least not in Cos-1 cells. Given that the Kozak region preceding the translation start of MR-B is weak, the existence of MR-B *in vivo* needs further clarification. Taken together, we conclude that the C allele increases MR-protein expression and thereby the *in vitro* transcriptional activity of the MR.

Finding genetic variants involved in the regulation of BP offers mechanistic insights into the development of hypertension in the general population and helps in identifying novel targeted therapeutic strategies to prevent cardiovascular disease. The physiopathological relevance of our *in vitro* results was tested by studying the association of the MRc.-2G>C polymorphism with parameters of BP regulation and electrolyte homeostasis in two cohorts under different experimental settings. Individuals carrying the CC genotype in either a healthy cohort under a high Na^+ - low K^+ diet or a mild hypertensive cohort had significantly lower plasma renin concentration/renin activity levels, respectively. In parallel with the lower renin levels, the CC individuals in the healthy group also presented lower plasma aldosterone levels. The observed lower active renin, total renin and lower aldosterone levels suggest a more efficient tubulo-glomerular feed-back in individuals with the CC genotype. This effect might be due to more efficient sodium reabsorption due to higher levels of MR in the distal tubule which was unmasked in conditions of low aldosterone synthesis and concentration, i.e. on a high Na^+ - low K^+ diet (Menard et al., 2006). Indeed the C allele results in higher MR levels after transfection in a kidney collecting duct epithelial cell line (RCSV3) or a kidney fibroblast-like cell line (COS). There were trends in similar directions for these parameters in the healthy group at baseline or during the low Na^+- high K^+ diet but this did not reach significance. The absence of significant difference between genotypes may be due to a low power of the study which was not initially designed to test the effect of the MR polymorphism. Finally, both in the mild hypertensive group and in a large Dutch cohort, male GG carriers had a higher SBP. Taken together, these results indicate that the CC carriers may have a more favourable cardiovascular profile as compared to the CG and GG carriers.

Aldosterone has emerged as a key hormone determining cardiovascular and renal damage and risk prognosis, in addition to its role in BP regulation and potassium and sodium homeostasis. Within the last ten years, blocking its effects with MR antagonists has been shown to have beneficial effects in congestive heart failure, especially after myocardial infarction, and proteinuric nephropathies (Mehdi et al., 2009; Pitt, 2005). Our data suggest that functional variants of the MR may be associated not only to different cellular responses to the hormone, but also, indirectly, to increased aldosterone levels that may activate both genomic and non-genomic pathways in non-epithelial target tissues to promote deleterious cardiovascular effects.

Only males carriers of the MRc.-2G>C GG genotype showed higher BP compared to males with the other genotypes in the mild hypertensive cohort, the polymorphism not being associated with

BP in women. The same gender dependent association was found with SBP in the large-scale Dutch cohort suggesting a sexual dimorphism. Many studies have reported gender-related differences in occurrence and severity of cardiovascular diseases, related to the hormonal status (Rosano et al., 2007). Interestingly, it has been shown that ovarian hormones positively affect salt sensitivity, protecting pre-menopausal women from the development of hypertension. After menopause, responsiveness of renin-angiotensin-aldosterone system changes, with a net increase in salt sensitivity (Schulman et al., 2006). In addition, cortisol responses to stressors are different between men and women and as discussed above, cortisol might have an effect on epithelial MR as well. We hypothesise that the effect of the MR c-2G>C G allele, which leads to less MR, correlates with higher blood pressure only in men and not in women because of the interference of ovarian hormones. To further elucidate the role of sex hormones or differences in cortisol levels a cohort containing post menopausal women should be tested for an association with this SNP.

In conclusion, the MRc.-2G>C C allele increases translation of the MR protein resulting in an increase in MR protein expression and a higher transactivational activity *in vitro*. In two independent studies, GG carriers of MRc.-2G>C showed higher renin and aldosterone levels compared to individuals with the other genotypes. Finally, male GG carriers showed higher SBP. We propose that the MR -2C variant could participate in protecting against high blood pressure

5

Human Mineralocorticoid Receptor (MR) gene haplotypes modulate MR expression and transactivation: implication for the stress response

Nienke van Leeuwen, Silja Bellingrath, E. Ronald de Kloet, Frans G. Zitman, Roel H. DeRijk, Brigitte M. Kudielka & Stefan Wüst

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Abstract

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in secretion of corticosteroids which facilitate behavioural adaptation. These effects exerted by corticosteroids are mediated by two brain corticosteroid receptor types, the mineralocorticoid (MR) receptor, with a high affinity already occupied under basal conditions and the glucocorticoid receptor (GR), with a low affinity only activated during stress.
Here, we studied MR gene haplotypes constituted by the two single nucleotide polymorphisms MR-2G/C (rs2070951) and MRI180V (rs5522). *In vitro* the haplotypes showed differences in cortisol-induced gene transcription and protein expression, while the structural variant MRI180V did not affect ligand binding.

Moreover, in a well characterized cohort of 166 school teachers these haplotypes have been associated with perceived chronic stress (Trier Inventory for the Assessment of Chronic Stress, TICS) and, in a subgroup of 47 subjects, with ACTH, cortisol and heart rate responses to acute psychosocial stress (Trier Social Stress Test, TSST). MR haplotypes were significantly associated with the TICS scales “excessive demands at work” and “social overload”. Subjects homozygous for haplotype MR-2C/MRI180, which *in vitro* showed highest expression and transactivational activity, displayed the highest salivary cortisol (p=0.000), plasma cortisol (p=0.010), plasma ACTH (p=0.003) and heartrate (p=0.018) responses.

It is concluded that the investigated MR haplotypes modulate cortisol-induced gene transcription *in vitro*. Moreover, these haplotypes may contribute to individual differences in perceived chronic stress as well as neuroendocrine and cardiovascular stress responses.

Key words: Mineralocorticoid Receptor (MR), genetic polymorphisms, stress

Introduction

Cortisol has profound effects in the brain, underlying behavioural adaptation to stress and feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis. These actions exerted by cortisol are mediated by a high affinity brain corticosteroid receptor, the mineralocorticoid receptor (MR; NR3C2) and a lower affinity glucocorticoid receptor (GR). The GR is widely expressed while the MR predominantly occurs in limbic brain areas including the hippocampus. Animal studies have shown that MR occupation is maintained at basal pulsatile cortisol levels, while the GR becomes only activated with rising cortisol levels in response to stress and at the peaks of the corticosterone pulses (Sarabdjitsingh et al., 2009; Lightman et al., 2008; Conway-Campbell et al., 2007). The MR and GR operate as transcription factors in the regulation of gene transcription, but recently these receptors were also found to mediate fast membrane-mediated actions (Karst et al., 2005; Di et al., 2003). Through the MR cortisol regulates basal HPA pulsatility (Atkinson et al., 2008) and the threshold or onset of the HPA axis response to stress (Wellhoener et al., 2004; Arvat et al., 2001; Dodt et al., 1993a; Ratka et al., 1989), while the GR facilitates the suppression of stress-induced HPA activation and promotes adaptation.

Two functional single nucleotide polymorphisms (SNPs) in the MR have been previously identified, namely MR-2G/C (rs2070951) located 2 nucleotides before the translation startsite and MRI180V (rs5522), a SNP resulting in an amino acid change in the N-terminal domain of the protein. Both SNPs affect transactivation *in vitro* (van Leeuwen et al., 2010; DeRijk et al., 2006). MR-2G/C is located outside the coding region of the MR but inside the Kozac translation regulatory sequence, and is expected to influence brain function via changes in MR protein expression. The structural variant MRI180V was previously found to be associated with HPA axis and autonomic nervous system reactivity (DeRijk et al., 2006). This effect exerted by MRI180V may occur through differences in ligand binding, translocation to the nucleus, dimerization or recruitment of coactivators. Furthermore, these two SNPs in the MR are in linkage disequilibrium resulting in three common haplotypes and one very rare (frequency less than 0.1%) haplotype (DeRijk et al., 2008). The *in vitro* and *in vivo* effects of these haplotypes are currently not known.

The main objective of the current study was to measure transactivation, ligand binding and protein expression of MRI180V, MR-2G/C and the resulting haplotypes. In addition, we sought to evaluate the association between these haplotypes and valid (endo)phenotypes for psychobiological stress regulation in a cohort that is independent of the samples that have previously been studied by our group (van Leeuwen et al., 2010; DeRijk et al., 2006). Therefore, we performed a genetic association analysis in a cohort of school teachers that has been characterized with the Trier Inventory for the Assessment of Chronic Stress (TICS) and the Trier Social Stress Test (TSST).

Materials and methods

Functional characterization *in vitro*

Construction of the hMR plasmids

The expression plasmid containing human MR was obtained from Dr. R. Evans (gene expression laboratory and HHMI, The Salk Institute for Biological Studies, La Jolla, Ca) and is described elsewhere (Arriza et al., 1987).

MR-2G/C (rs2070951) and MRI180V (rs5522) sites were mutated from G to C and from A to G, respectively using primers 5'-GGCCGAGGCAGCGATGGAGACCAAAG-3' and 5'-CGCTGCCTCGGCCCTTTGGTCTCCAT-3' and primers 5'-GGCGTCATGCGCGCCGTTGTTAAAAGCCCCTAT-3' and 5'-ATAGGGCTTTTAACAACGGCGCGCATGACGCC-3' and the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol.

After mutagenesis the hMR insert of the plasmid was sequenced to assure absence of other mutations.

Transactivation assay

Cos-1 cells (African green monkey kidney cells) were cultured in DMEM high glucose supplemented with 10% FCS (Gibco, Paisley, UK). Cells were seeded in 24-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 3 x 10^4 cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using SuperFect (Qiagen, Venlo, The Netherlands). hMR plasmids and the reporter pasmid TAT3-Luc (tyrosine amino transferase triple hormone response element) were used at 100 ng/well. The control plasmid pCMV-R (Promega, Leiden, The Netherlands) coding for Renilla luciferase controlled by cytomegalovirus (CMV) promoter was used (10 ng/well). One day after transfection, the cells were treated with cortisol (Sigma-Aldrich, Zwijndrecht, the Netherlands) in concentrations ranging from 0 to 10^{-8} M. After 24h of incubation the cells were harvested in passive lyses buffer (Promega) and firefly and Renilla luciferase activity was determined using a dual label reporter assay (Promega) and a luminometer (CENTRO XS3 LB960, Berthold, Bad Wildbad, Germany). Three separate experiments were performed and all three experiments were performed in triplicate.

Western blot

For western blot Cos-1 cells were seeded in 6-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 2x10^5 cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using Trans-it Cos transfection reagent (Mirus, Madison, USA). Plasmids containing one of the hMR variants or no hMR (control) were used at 2µg/well. Cells were harvested 48 hours after transfection. The primary antibody MR 1D5 (a generous gift by Gomez-Sanchez, Division of endocrinology, University of Mississippi, Jackson, MS) was diluted 1:1000 in 0.5 % milk powder in Tris buffered saline and Tween 20 (TBST) and incubated for 1h at room temperature (RT). The secondary antibody goat anti-mouse IgG HRP was used in 1:5000 dilutions in TBST with 0.5 % milk for 1 h at RT. Tubulin was used as a control for the amount of cells and the monoclonal anti γ-Tubulin was used at a 1:1000 dilution (T6557; Sigma-Aldrich, Zwijndrecht, the Netherlands). The ECL detection system (GE healthcare, Diegem, Belgium) was used for detection. The differences in intensity of the MR bands were quantified with Image J

(ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). Three separate experiments were performed.

Ligand binding assay

Cos-1 cells were seeded in 20 cm plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at $2x10^6$ cells/plate in DMEM supplemented with 5% charcoal-stripped serum. Cells were transfected the next day using Mirus TransIt- COS reagent according to the manufacturer's protocol (Sopachem, Ochten, The Netherlands) and hMR plasmids were used at 30 µg/plate. After 24 hours medium was replaced with serum free DMEM and after another 24 hours cells were pelleted. All further steps are carried out at 0°C. Cells were resuspended in 3.5ml buffer (5mM Tris-HCl (pH 7.4), 1mM EDTA, 1mM B-Mercaptoethanol, 10mM Na-Molybdate, 5% glycerol) per plate and 3 x 15 seconds homogenised using an electric homogenizer (Pro200, Pro scientific, Oxford, CT, USA). The homogenate was centrifuged (100.000 x g, 2°C) to obtain cytosol.

200µl cytosol was incubated with [^{3}H]Cortisol (70 Ci/mmol, Amersham, Buckinghamshire, UK) to asses total binding or [^{3}H]Cortisol and a 500 fold excess of dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands) to asses non-specific binding. [^{3}H]Cortisol was used at 0.5nM, 1nM, 1.5nM, 2.5nM, 3.5nM, 5nM. After vortexing and 3 hours incubation on ice bound and free [^{3}H]Cortisol fractions were separated by Sephadex LH-20 as described previously (de Kloet et al., 1975). Fractions containing the receptor bound radioligand were collected, vortexed with 3ml Ultima Gold scintillation fluid (Perkin Elmer, Waltham, Massachusetts, USA) and radioactivity was measured in a liquid scintillation analyzer (1900CA Packard, Perkin Elmer). Three separate experiments were performed and all three experiments were performed in triplicate.

Statistical analysis

The *in vitro* experiments were analyzed using GraphPad prism 4 (GraphPad software Inc, San Diego, CA). In the transactivation assays firefly/renilla luciferase ratios were normalized against the highest signal and background expression was subtracted. MR protein expression measured by western blot was normalized against γ-Tubulin. The differences between the four hMR variants were analyzed with one and two-way ANOVAs with Bonferroni posttests. In the radioligand binding assay one-binding-site curve fitting was used to determine the dissociation constant (Kd) and maximal binding (Bmax). The specific MR cortisol binding was obtained by subtracting the non-specific binding from the total binding. The difference in Kd and Bmax between MRI180 and MR180V was tested with a t-test. *In vitro* results are shown as the mean ± SD.

Genetic association study

Recruitment

We approached teachers of all major school types in the region of Trier (Germany) and Luxembourg by means of personal visits in local schools and by newspaper announcements. Teachers were entered into the study if they reported to be free of psychiatric disorders, diabetes, pregnancy, and corticosteroid or psychotropic medication. Written informed consent was obtained from all participants and the protocol was approved by the ethics committee of the University of Trier and the Rheinland-Pfalz State Medical Association.

DNA extraction and Genotyping

DNA was extracted from 10ml peripheral venous blood following a standard method (Miller et al., 1988). Subjects were genotyped for the MR-2G/C and MRI180V SNPs by both matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc., San Diego, CA, USA) and by TaqMan pre-designed SNP genotyping assays, assay ID C12007869_20 and C1594392_10, respectively, in combination with TaqMan universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Reaction components and amplification parameters were based on the manufacturer's instructions. Genotyping the samples with two different genotyping methods decreases method specific genotyping errors.

Assessment of perceived chronic stress

Perceived chronic stress was measured using the short version of the Trier Inventory for the Assessment of Chronic Stress (TICS-S) (Schulz and Schlotz, 1999). The TICS covers nine dimensions of chronic stress, namely work overload, social overload, excessive demands at work, lack of social recognition, work discontent, social tension, performance pressure, social isolation and chronic worrying. For each item, the frequency of the experience in the last year had to be indicated on a 5-point rating scale, ranging from "never" to "very often."

Psychosocial stress protocol

The Trier Social Stress Test (TSST) consists of a three minutes preparation phase followed by a five minutes free speech phase (job interview) and a five minutes mental arithmetic task in front of a panel and a camera (for a detailed description of this protocol see (Kudielka et al., 2007a; Kudielka et al., 2007b). Test sessions were only run in the afternoon, starting between 1500h and 1600h. Participants were instructed to refrain from physical exercise, a heavy lunch and alcoholic beverages on test days. Premenopausal women not taking oral contraceptives were invited during the luteal phase of the menstrual cycle. The menstrual phase was estimated on the basis of the first day of last menses and the subject's usual cycle length. Only women with a regular cycle between 28 and 35 days were included and the luteal phase was defined as the last 14 days of the cycle. In the laboratory, at first an intravenous catheter was inserted in the antecubital vein of the dominant arm for later blood draws and subjects were instrumented with heart rate monitors. Heart rate was measured at 5 second intervals using a transmitter belt with a wrist receiver (Polar Sport Tester; Polar Electro, Büttelborn, Germany). After a rest period of 40 min following canula insertion and 10 min before the start of the stressor, subjects were asked to stand up. After TSST exposure subjects remained in an upright position for another 10 minutes.

Blood and Saliva Sampling

Blood samples for the assessment of ACTH and total plasma cortisol were collected in EDTA containing monovettes (Sarstedt, Nümbrecht, Germany)

1 min before as well as 1, 10, 20, 30 and 90 min after cessation of the TSST. In parallel, subjects obtained native saliva in 2 ml reaction tubes (Sarstedt, Nümbrecht, Germany) for later assessment of salivary cortisol. Additional saliva samples were obtained at 45 and 60 min after cessation of the TSST.

Biochemical analysis

Salivary cortisol was measured by an in-house DELFIA (intra- and inter-assay variation ≤11.5%). Blood samples were instantaneously stored on ice and centrifuged at 4ºC for 15 min at 2000 g and pipetted into aliquots. Aliquots for the analysis of plasma cortisol as well as saliva samples were stored at -20°C and aliquots for the analysis of ACTH were stored at -80°C until assayed. ACTH and total plasma cortisol were measured by ELISA assays (plasma cortisol: IBL Hamburg, Germany, intra- and inter-assay variation ≤6.9%; ACTH: Biomerica Newport Beach, USA, intra- and inter-assay variation ≤6.0%).

Statistical analysis

Haploview (Barrett et al., 2005a) was used to calculate Hardy Weinberg equilibrium (HWE) and linkage disequilibrium among the two MR SNPs (estimated with D' and r^2). Haplotypes were estimated and assigned to each individual using SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/). In order to analyze the association between haplotypes and perceived chronic stress levels, we used the haplotype trend regression (HTR) approach as outlined by Zaykin et al (Zaykin et al., 2002). Assuming additive effects of the haplotypes on the trait, the HTR approach tests for the contribution of individual haplotypes rather than haplotype pairs. We applied a permutational approach to obtain empirical p-values utilizing the HTR function of the R-package "gap", version 1.0-17 (R 2.7.2; http://www.R-project.org) with 10.000 simulations. HTR procedures provide a global p-value as well as p-values indicating the association between the trait and each haplotype. A two-stage strategy was applied to test for possible associations between haplotypes and neuroendocrine as well as autonomic TSST responses. First, the HTR approach was used as global significance test. Therefore, area under the response curve (AUC) measures were computed for salivary cortisol, plasma cortisol, ACTH and heart rate responses and entered into the HTR models. Secondly, *post hoc* tests were performed to further inspect the detected effects. To use the full information of the repeated measures design this was done with general linear models (GLMs) to assess the repeated measures effect *time*, the between-subjects effect *haplotype* as well as the interaction *time x haplotype*. In order to control for possible influences of gender, sex was included as additional predictor. Effect sizes were calculated for significant results by partial eta squared (η^2). Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. GLM procedures were performed using the PASW statistical software package (Version 18.0). Unless otherwise stated, results are expressed as mean ± standard error of the mean (S.E.M.). While cortisol, ACTH and heart rate values were log-transformed before statistical analyses to yield unskewed outcome variables, figures show untransformed means in order to provide a more naturalistic impression of endocrine levels.

Results

Functional characterization *in vitro*

All four MR haplotypes were tested *in vitro.* According to the observed frequency in the population (DeRijk et al., 2008) the haplotypes are referred to as Hap 1 (GA), constituted by MR-2 G and MRI180V A, Hap 2 (CA), constituted by MR-2 C and MRI180V A, Hap 3 (CG), constituted by MR-2C and MRI180V G and the *in vivo* rarely observed Hap 4 (GG), constituted by MR-2 G and MRI180V G.

Transactivation assay

The four different MR haplotypes showed differential cortisol-induced luciferase transcription from a triple tyrosine amino transferase (TAT-3) promotor ($F_{3,26}$= 42.7; p<0.0001; η^2= 0.06; Figure 1). The analysis of the dose response curves revealed a significant difference in the EC50 between the four MR haplotypes; Hap 1 (GA) EC50= $3.9x10^{-11}$, Hap 2 (CA) $1.7x10^{-11}$, Hap 3 (CG) $1.9x10^{-11}$ and Hap 4 (GG) $7.3x10^{-11}$ ($F_{3,44}$= 1651; p<0.0001; η^2= 0.99) but no difference in the slope of the curves. Hap 1 (GA) and 4 (GG), the two haplotypes containing MR-2 G showed a significant lower maximal luciferase expression (Emax) than Hap 2 (CA) and 3 (CG), i.e. the two haplotypes containing MR-2 C ($F_{3,28}$= 29.2; p<0.0001; η^2= 0.76). Although the effect on transactivation was largest for the MR-2G/C SNP, MRI180V also influenced the transactivation with the A (MRI180) having a lower EC50 than the G (MR180V).

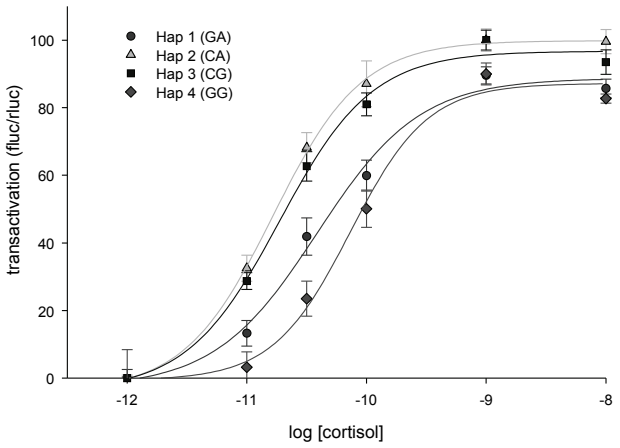


Figure 1. Cortisol induced transactivation of the four MR haplotypes on a TAT-3 promoter in Cos-1 cells. Cortisol concentrations are indicated in log units and responses are displayed as reporter (fluc)/control (rluc) ratios. The four haplotypes showed significantly different responses (p<0.0001), with Hap 2 (triangle) being most efficient followed by resp. Hap 3 (square), Hap 1 (circle) and Hap 4 (diamond). Figure 1 represents the data of three separate experiments, which did not show significant differences when compared to each other, and were therefore pooled.

Western blot

The MR haplotypes influenced MR protein expression in transfected COS-1 cells ($F_{3,4}$= 7.07; p=0.03; η^2= 0.80, Figure 2). *Post hoc* analysis revealed that protein expression was only influenced by MR-2G/C and not by MRI180V. Hap 2 (CA) and 3 (CG), the two plasmids containing MR-2C, showed higher MR protein expression compared to Hap 1 (GA) and 4 (GG), the two plasmids containing MR-2 G (all combinations p<0.05), while there was no significant difference between Hap 1 and 4 and between Hap 2 and 3.

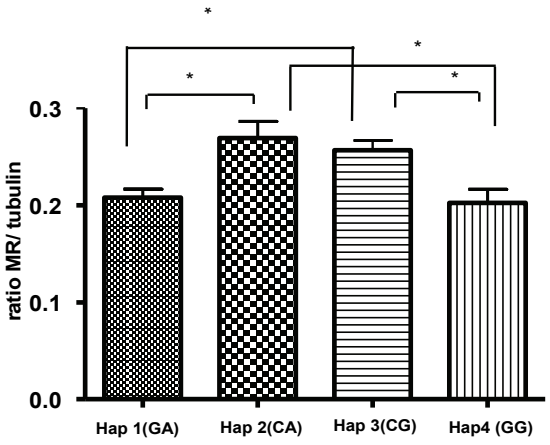


Figure 2. MR protein expression measuered in gray values on a western blot normalized against tubulin measured in gray values. The haplotypes Hap 2 and 3 had significantly higher MR expression than Hap 1 and 4 (* p<0.05) while there was no significant difference between Hap 1 and 4 and between Hap 2 and 3.

Ligand binding

Cortisol binding to the MR (Kd and Bmax) was not influenced by MRI180V. The Kds of MRI180 and MR180V were not significantly different, being 0.86± 0.20 and 0.93±0.16 nM, respectively. There was also no significant difference in Bmax, showing values of 6539 ±499 and 7112±371 binding sites/cell for the MRI180 and MR180V, respectively. As there was no significant difference between the three separate experiments, data of the three experiments were pooled for analysis.

Genetic association study

Genotypes and haplotypes

The two employed genotyping methods yielded identical results and there were no drop outs. The distribution of both SNPs, MRI180V and MR-2G/C, did not deviate significantly from Hardy Weinberg equilibrium (HWE) (p=0.7 and p=0.2 resp). The estimated linkage between MRI180V and MR-2G/C was D'=1 (conf bounds 0.63-1) and r^2=0.093. As expected, in this sample Hap 1 (GA) showed with 48.8% the highest frequency followed by Hap 2 (CA) with a frequency of 41.9% and Hap 3 (CG) with a frequency of 9.3%. Consistent with previous studies Hap 4 (GG) was not observed in this cohort (see Figure 3). One subject showed the very rare genotype CGCG (i.e. homozygous for Hap 3) and was excluded from all association analyses.

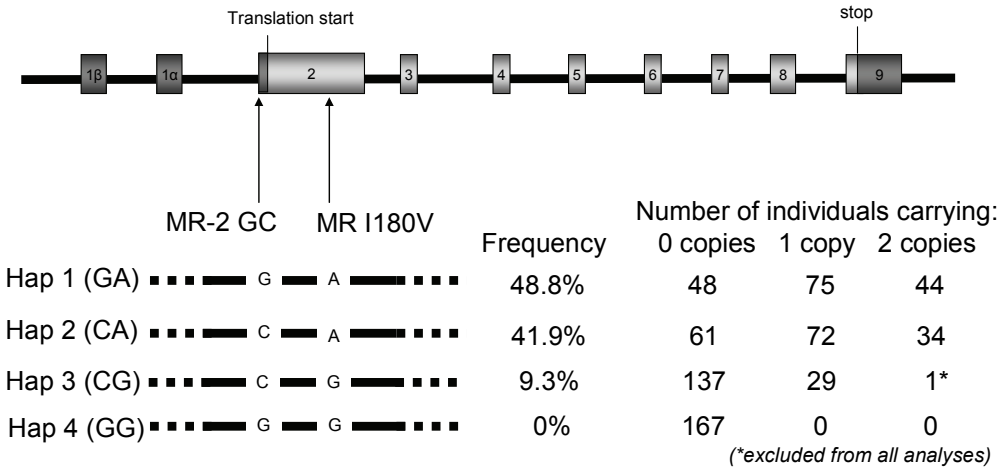


Figure 3. Schematic overview of the human MR gene (not on scale) with the location of the MR SNPs MR-2G/C and MRI80V, the haplotypes and frequencies of the haplotypes formed by these SNPs. Dark gray boxes represent untranslated exonic regions, light gray boxes represent translated exonic regions and the black line represents the intronic regions of the gene. MR-2G/C is located in the untranslated exonic region just 2 nucleotides before the translation start and MRI180V is located in the translated region of exon 2. The frequency refers to the haplotype frequency observed in this cohort and the number of individuals in this cohort carrying 0, 1 (heterozygotes) or 2 (homozygotes) copies of a haplotype is indicated.

Final sample

The sample for the present analysis consisted of 166 healthy subjects (55 males and 111 females). Participants were between 23 to 63 years of age (mean age: 45.58 ± 9.8) and had a mean body mass index (BMI) of 25.9 ± 4.7. Fifteen of the subjects reported to be smokers. Questionnaire data from 163 to 166 participants (due to a different number of missing values across scales) could be analyzed. Gender was included in all statistical analysis but there were no gender-haplotype interactions observed in any of the associations. Therefore we do not show the results of this predictor.

Perceived chronic stress

HTR models revealed associations between the MR haplotype structure and perceived chronic stress assessed with the TICS in respect to four subscales, namely "social overload", "excessive demands at work", "social tension", and "social isolation" (Table 1). While global p-values were significant for "social overload" (F=3.21, p=0.042) and "excessive demands at work" (F=3.65, p=0.029), a trend was detected for "social tension" (F=2.39, p=0.095) and "social isolation" (F=2.63, p=0.076). Although the HTR approach controls for multiple comparisons of haplotype pairs within one TICS scale, it does surely not control for multiple testing across all nine TICS subscales. None of the significant global p-values would withstand such correction but nonetheless we performed post-hoc tests, in order to illustrate the direction of these exploratory results. Inspection of haplotype specific p-values for these four scales suggested that carriers of Hap 3 (CG) reported more chronic stress in terms of "excessive demands at work" (F=7.27; p=0.008) and "social overload" (F=4.17; p=0.045) than non-carriers. Furthermore, individuals with two copies of Hap 1 (GA) seemed to report more chronic stress in terms of "social isolation" (F=4.93; p=0.029) and "social tension" (F=4.80; p=0.032) than individuals with one copy or zero copies of Hap 1. Consistently, individuals with 2 copies of Hap 2 (CA) seemed to score lower on "social isolation" (F=4.95; p=0.027).

Table 1. Association between subscales of the Trier Inventory for the Assessment of Chronic Stress and MR haplotypes.

	MR Haplotypes	0 Copies	1 Copy	2 Copies	Global Test p [F]	Haplotype Specific Test p [F]
		Mean (± Std)				
Work Overload					n.s.	
	GA	2.22 (0.91)	2.29 (0.81)	2.41 (0.98)		
	CA	2.48 (0.94)	2.21 (0.82)	2.19 (0.89)		
	CG	2.26 (0.88)	2.51 (0.88)			
Social Overload					**.042** [3.21]*	
	GA	1.93 (0.91)	1.91 (0.94)	2.10 (0.87)		n.s.
	CA	2.18 (0.96)	1.84 (0.85)	1.85 (0.90)		**.056** [3.75]$^{+}$
	CG	1.90 (0.86)	2.28 (0.91)			**.045** [4.17]*
Excessive Demands at Work					**.029** [3.65]*	
	GA	1.35 (0.77)	1.19 (0.79)	1.29 (0.80)		n.s.
	CA	1.37 (0.86)	1.18 (0.75)	1.25 (0.73)		n.s.
	CG	1.19 (0.91)	1.62 (0.79)			**.008** [7.27]*
Lack of Social Recognition					n.s.	
	GA	1.60 (0.99)	1.63 (1.03)	1.84 (1.10)		
	CA	1.73 (1.12)	1.66 (1.02)	1.61 (0.95)		
	CG	1.71 (1.02)	1.50 (1.13)			
Work Discontent					n.s.	
	GA	0.95 (0.90)	0.99 (0.77)	1.12 (0.76)		
	CA	1.06 (0.72)	1.04 (0.86)	0.87 (0.82)		
	CG	1.01 (0.80)	1.00 (0.83)			
Social Tension					**.095** [2.39]$^{+}$	
	GA	1.13 (0.72)	1.17 (0.72)	1.45 (0.78)		**.032** [4.80]*
	CA	1.44 (0.77)	1.10 (0.72)	1.21 (0.70)		**.071** [3.44]$^{+}$
	CG	1.26 (0.74)	1.16 (0.79)			n.s.
Performance Pressure					n.s.	
	GA	1.84 (0.70)	1.79 (0.74)	1.89 (0.70)		
	CA	1.91 (0.77)	1.77 (0.68)	1.83 (0.70)		
	CG	1.82 (0.69)	1.91 (0.84)			
Social Isolation					**.076** [2.63]$^{+}$	
	GA	1.28 (0.86)	1.71 (0.95)	1.73 (1.10)		**.029** [4.93]*
	CA	1.73 (1.02)	1.64 (0.95)	1.25 (0.87)		**.027** [4.95]*
	CG	1.59 (0.99)	1.69 (0.88)			n.s.
Chronic Worrying					n.s.	
	GA	1.89 (1.02)	1.68 (0.84)	1.78 (1.09)		
	CA	1.80 (1.04)	1.74 (0.96)	1.75 (0.83)		
	CG	1.70 (0.92)	2.04 (1.15)			

*(Table shows asymptotic F- and empirical p-values; *p<.05, $^{+}$p<.10)*

ACTH, cortisol and heart rate responses to acute psychosocial stress

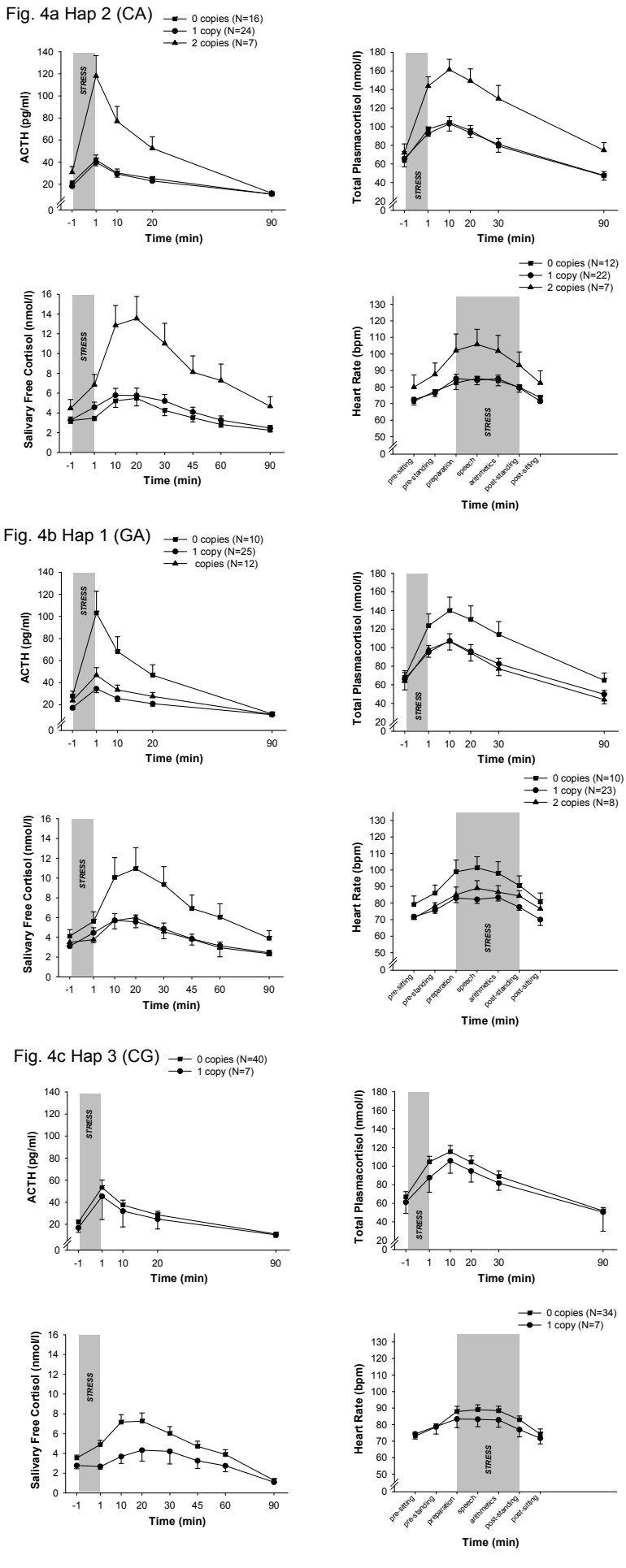
A subsample of 54 participants (20 males and 34 females) agreed to also participate in the acute stress protocol. Participants of this subsample were between 25 to 63 years of age (mean age: 48.56 ± 8.8) and had a mean body mass index (BMI) of 25.9 ± 4.8. Haplotype frequencies of this subsample were very similar to frequencies in the main sample. Because of the well-known

intervening effects of oral contraceptive or sex steroid intake (Kudielka et al., 1999; Kirschbaum et al., 1999) as well as smoking (Rohleder and Kirschbaum, 2006) on acute HPA axis stress responses, we excluded three women taking oral contraceptives or receiving hormonal replacement therapy and two smokers from all further analyses. Two further subjects had missing data in the endocrine measures while six subjects had missing heart rate data due to technical problems. Thus, we included 47 subjects in the final analysis of endocrine and 41 subjects in the analysis of heart rate responses.

Despite the small size of this subsample MR haplotypes were significantly associated with neuroendocrine and autonomic TSST responses in a rather consistent way. Regarding the global test HTR procedures revealed significant associations between the investigated MR haplotype structure and the area under the curve measures for salivary cortisol responses (F=6.80; p=0.005), plasma cortisol responses (F=3.34; p=0.046), and ACTH responses (F=4.03; p=0.029). The respective effect for heart rate responses showed a trend towards statistical significance (F=2.37; p=0.109).
To use the full information of the repeated measures design, *post hoc* inspection of associations of specific haplotypes was done with general linear models. For Hap 2 (CA), significant main effects *haplotype* were observed for ACTH ($F_{2,41}$=6.69, p=0.003, η^2=0.25), plasma cortisol ($F_{2,41}$=5.12, p=0.010, η^2=0.20), salivary cortisol ($F_{2,41}$=12.11, p=0.000, η^2=0.37) as well as heart rate ($F_{2,35}$=4.51, p=0.018, η^2=0.21). Across all measures, individuals with two copies of Hap 2 showed a stronger response to the stressor than individuals with one copy or zero copies. In addition, significant *time x haplotype* interactions were found for ACTH ($F_{3.76,76.39}$=4.58, p=0.003, η^2=0.18) and salivary cortisol ($F_{6.89,141.17}$=2.57, p=0.017, η^2=0.11), while the respective interactions for plasma cortisol and heart rate were not significant (all p>.14). Mean responses are shown in Figure 4a.

A similar picture emerges for Hap 1 (GA), which is not surprising given that Hap 1 and Hap 2 are largely complimentary. Here, those individuals with zero copies of Hap 1 showed significantly elevated ACTH (main effect $F_{2,41}$=7.73, p=0.001, η^2=0.27), salivary cortisol (main effect $F_{2,41}$=6.67, p=0.003, η^2=0.25) and heart rate (main effect $F_{2,35}$=4.96, p=0.013, η^2=0.22) levels. The effect for plasma cortisol levels just missed the level of significance (main effect $F_{2,41}$=2.90, p=0.066). A significant *time x haplotype* emerged for ACTH ($F_{3.61,74.05}$=4.68, p=0.003, η^2=0.19) and a trend was observed for salivary cortisol ($F_{3.26,128.26}$=1,91, p=0.072), while the respective interactions for plasma cortisol and heart rate were not significant (all p>.19, Figure 4b). Finally, we did not detect a significant association between Hap 3 (GC) and neuroendocrine and autonomic TSST responses (p>.10 for all main effects *haplotype* and p>.15 for all interactions *time x haplotype,* Figure 4c).

Figure 4. ACTH , total plasma cortisol , salivary cortisol and heart rate responses to psychosocial stress (TSST) in subjects carrying 0, 1 or 2 copies of haplotype a) CA, b) GA and c) CG; data are expressed as mean ± S.E.M.



Discussion

Here we described neuroendocrine and behavioral consequences of two common functional polymorphisms in the human MR, MRI180V and MR-2G/C, both *in vitro* and *in vivo*. The haplotypes of the two SNPs showed differences in cortisol-induced transcription of the reporter gene. From protein analysis of the haplotypes it can be concluded that MR-2G/C changes protein expression while MRI180V did not have this effect. Furthermore, MRI180V did not affect ligand binding. Our data suggest that the haplotypes are associated with stress-induced HPA axis and autonomic responses following a psychosocial stress test. Moreover, the haplotypes might be associated with several aspects of perceived chronic stress.

Although transactivation assays have been performed with the two individual MR SNPs before (van Leeuwen et al., 2010; DeRijk et al., 2006), the combinations of the two SNPs, as occur *in vivo* as part of the observed haplotypes, have not been tested so far. It might be possible that the SNPs interact, resulting in an additional effect of the haplotypes (e.g. not effect size 2 = effect size 1 + effect size1 but effect size 1 + effect size1 = effect size >2 or <2), therefore it is important to test haplotypes in addition to single SNPs. However, in this study statistical analysis did not reveal an interaction effect between the -2G/C and the MRI180V.

MRI180V produces an amino acid change in the N-terminal domain, which is involved in recruiting co-regulators that selectively modulate transcriptional activity of the MR. As shown in the current study, this effect was not mediated by differences in cortisol binding characteristics, since no differences in maximal binding capacity (Bmax) or dissociation constants (Kd) were observed between MRI180 and MR180V. This suggests that other factors such as differences in translocation to the nucleus, dimerization of the MR or binding of co-regulators might be responsible for the observed differences in transactivation.

In contrast to the MRI180V, the MR-2 G/C is not changing the primary structure of the receptor and is therefore less likely to have an effect on MR protein characteristics. In this study we showed that both haplotypes containing MR-2 C had a higher MR protein expression as compared to the two haplotypes containing MR-2 G while the MRI180V did not influence the protein expression. This finding explains the higher transactivational capacity of the two haplotypes containing MR-2 C, as occurring in haplotypes 2 and 3. In a supplementary part of the present study we investigated the association between these MR gene variants and subjectively perceived chronic stress and neuroendocrine as well as autonomic responses to acute experimental psychosocial stress. We selected a small but well characterized sample of healthy school teachers, since the teaching profession has been repeatedly described as a potentially stressful occupation (Guglielmi and Tatrow, 1998), which is reflected in high rates of early retirement among German school teachers (Weber, 2004). Furthermore, it is important to note that this cohort is independent of the samples in which the previously reported associations between MR gene polymorphisms and HPA axis regulation have been observed (van Leeuwen et al., 2010; DeRijk et al., 2006). The clear weakness of this cohort is its rather modest sample size and this holds in particular for the subsample that was exposed to the TSST also the lack of a second cohort to confirm the findings is a limitation of the study. Therefore, all reported association findings have an exploratory character. However, given this limitation, the observed associations between MR gene haplotypes and biological stress

responses have been remarkably consistent across the different indices. Individuals carrying two copies of haplotype 2 (CA) showed higher salivary cortisol, plasma cortisol, ACTH as well as heart rate responses to acute psychosocial stress, compared to individuals with only one or zero copies of this haplotype. Despite the small sample at least the global effect for salivary cortisol responses did survive bonferroni correction for multiple comparisons (corrected for four HTR procedures) and some of the GLM p-values are remarkably small. The distinct mean ACTH and cortisol response differences shown in Figure 4a and 4b were not caused by single subjects with extreme response patterns. As a further consequence of the sample size it was not possible to compute a separate analysis for males and females. We did, however, control for sex effects statistically, we did only include females who did not take oral contraceptives and premenopausal females were tested in the luteal phase of the menstrual cycle.

The association between MR gene haplotypes and perceived chronic stress could be investigated in a larger, but still modest sample of 166 subjects. HTR models revealed significant associations between the MR haplotype structure and the TICS subscales “social overload” and “excessive demands at work”. On the one hand, these effects surely have to be interpreted with caution as the TICS comprises nine subscales and the observed effects do not survive a statistical correction for multiple testing.

On the other hand stress is conceptualized as a multidimensional construct arising from person–environment interactions (Mcewen and Stellar, 1993; Lazarus and Launier, 1978) and different stress dimensions are assumed to be influenced by personality and other trait factors to different degrees, which in turn could explain different genetic effects. In line with this speculation, in a twin study (Federenko et al., 2006) we found clearly differing heritabilities varying between 5% and 45% for different stress scales. It appears plausible, that associations between genetic polymorphisms become visible for some facets of perceived chronic stress, but not for others. Therefore, our results suggest an association between MR gene haplotypes and some aspects of perceived chronic stress, but this finding needs to be confirmed in a larger sample.

Combining the neuroendocrine and perceived chronic stress data, haplotype 2 appears to be associated with higher neuroendocrine stress-responses and better stress handling. A previous study showed that the MR -2 C variant associates with lower basal non-stress levels of cortisol in an elderly population (Kuningas et al., 2007). Thus one can speculate that a more reactive HPA axis with lower basal cortisol levels is beneficial for coping with stressors, as has been proposed (de Kloet et al., 2007). Moreover, the *in vitro* data show that haplotype 2 increases MR-expression, again adding to the notion that higher MR-expression is beneficial. This is further substantiated by animal research showing that increased MR-expression in the forebrain of mice results in less anxiety-like behavior (Rozeboom et al., 2007). With respect to the HPA axis response, the MR is involved in tonic inhibition of cortisol / corticosterone levels. Furthermore, during the ageing process, a loss of MR-expression in the brain is observed which coincides with less sensitivity towards ACTH in the Brown Norway rat (Van Eekelen et al., 1992). Also in MR forebrain knock out mice, less adaptation of the HPA axis response to stress is observed (Brinks et al., 2009). This indicates that higher MR-expression in the brain leads to a more dynamic HPA axis response with lower basal non-stress levels. The precise mechanism how the putative increased MR-expression

leads to a more reactive HPA axis responses and resilient behavior to stressors is unknown. MR-expression is essential for neuronal protection and stability of neuronal circuits (Lai et al., 2009; de Kloet et al., 2007).

Haplotype 3 is the only haplotype containing the MR180V (G). The mechanism behind the observed decrease in transactivation with this allele is not yet elucidated, this polymorphism causes an aminoacid change in the protein but ligand binding and protein expression are not influenced, this makes it difficult to speculate about a mechanism in vivo. We previously showed in a different cohort that the G allele carriers have higher TSST responses however in this cohort MR180V (G) seems to have no effect on the TSST response since the largest difference is observed between hap 1 and hap 3. Furthermore MR180V (G) has previously been associated with more feelings of depression in elderly while we see lower scores on the stress scales with hap 3. Our group sizes are small therefore our finding is purely explorative and more research should be performed to first elucidate the molecular mechanism of MRI180V and secondly the effect on perception of stress and the consequences.

The recent discovery of a MR located in the membrane, in addition to the nuclear MR, has further implications (Karst et al., 2005). This low affinity membrane form of the MR becomes activated during stress-levels of cortisol and increases excitatory glutaminergic transmission while decreasing post-synaptic after-hyperpolarization (Joels et al., 2008). This rapid excitatory MR-mediated effect may very well underlie the non-genomic actions exerted by cortisol on neuroendocrine, emotional and cognitive processes (Brinks et al., 2009). Therefore, it will be a challenge for future research to dissociate during a psychosocial stressor the genomic and non-genomic effects mediated by the MR on processing of stressful information resulting in HPA axis reactivity and behavior. The MR haplotypes identified in this study may be very helpful in this respect.
In conclusion, *in vitro* assays demonstrate large differences in transactivation between the haplotypes. The molecular mechanism of these differences is only partly elucidated. *In vivo,* individuals with two copies of MR haplotype 2 (CA) had the most dynamic response to an acute psychosocial stressor, both the HPA axis and autonomic responses were higher in these individuals. Furthermore, our data suggest involvement of MR gene variants in perceived chronic stress. All together, it is concluded that these MR haplotypes may contribute to individual differences in the neuroendocrine response during coping with psychological stress.

6

Two haplotype blocks in the Mineralocorticoid Receptor (MR) associate with neuroticism but not with mood and anxiety disorders

N. van Leeuwen, M.D. Klok, G. Veen, E.R. Klaassens, E.R. de Kloet, F.G. Zitman and R.H. DeRijk

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Summary

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA)-axis, resulting in the secretion of cortisol, which feeds back on stress-induced HPA activation and facilitates behavioral adaptation. These effects exerted by cortisol are mediated by two brain corticoid receptor populations, i.e. the mineralocorticoid receptors (MR) and the glucocorticoid receptor s(GR), MR and GR operate in complementary fashion in control of the HPA axis and behavior. MR is mainly expressed in limbic brain structures regulating predominantly the initial reaction to psychosocial stressors, which is subsequently suppressed by the ubiquitous present GR.

Dysregulation of HPA-axis reactivity has frequently been reported in patients with mental disorders. In addition, the personality trait "neuroticism" is regarded as a vulnerability factor for depression. Twin studies demonstrated that cortisol levels, psychopathology and neuroticism are heritable. Previously, it was demonstrated that genetic variation in the MR is functional *in vitro* and influences responses to a psychosocial stressor.

In the current study, involvement of the MR in neuroticism and psychopathology was tested by measuring common genetic polymorphisms in the MR-gene in a cohort of healthy controls (n=50) and patients with mood and/or anxiety disorders (n=100). In this relatively small cohort there was no difference in MR genotype distribution between patients and controls. However, the genetic variation in the MR appeared to be associated with neuroticism in patients (p=0.02).

In conclusion, these preliminary findings suggest that genetic variation in the MR associates with neuroticism, indicating involvement of the MR in human behavior. This finding is in support of previous evidence from animal studies linking variation in MR function with emotional and cognitive aspects of the stress response.

Introduction

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA)-axis, resulting in the secretion of cortisol, which facilitates behavioral adaptation. Dysregulation of the HPA-axis has frequently been reported in patients with mental disorders. Patients with severe depression often have elevated cortisol levels. Furthermore, the personality trait "neuroticism" that is characterized by chronic negative affect is regarded as a vulnerability factor for depression (Weinstock and Whisman, 2006; Ormel et al., 2004; Clark et al., 1994).

Although the magnitude of heritability differs largely between studies, it is clear that cortisol levels, mood and anxiety disorders and neuroticism are heritable (reviewed by (Flint, 2004). In addition several studies showed that cortisol levels are positively correlated with neuroticism. Therefore genetic variability in factors regulating the stress system might underlie differences in neuroticism and susceptibility to psychopathology.

Central in the regulation of the HPA-axis by cortisol are two brain corticoid receptor population, the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). GR is expressed throughout the whole body and mediates via the elevated cortisol levels the metabolic, immunological, cardiovascular and behavioural adaptations in response to stress. MR binds in the limbic brain not only cortisol but also aldosterone with high affinity, but since the concentration of cortisol is much higher the brain predominantly *sees* the latter steroid. In aldosterone target tissues such as sweat glands, distal colon, kidney and salivary glands cortisol is converted to inactive cortisone by 11β hydroxy steroid dehydrogenase type 2. Brain MR is in particular involved in the regulation of basal HPA-axis pulsatility and in the onset of the stress response.

Several studies using animal models have demonstrated MR-mediated effects on behaviour. MR blockade clearly influenced coping with stressful challenges (Oitzl and de Kloet, 1992), observed as a change in the initial search strategy for an escape route in the Morris water maze. Other studies demonstrated that the MR blockade inhibits the onset of aggressive behaviour in male rats (Haller et al., 1998) and rainbow trout (Schjolden et al., 2009). Furthermore, transgenic mice with increased levels of MR in the forebrain showed decreased anxiety-like behaviour and enhanced memory (Rozeboom et al., 2007; Lai et al., 2007). In humans, MR-blockade impaired e.g. selective attention and mental flexibility, behavioural responses that represent the inital behavioural response to a stressor (Otte et al., 2007).

Involvement of genetic variation in the human MR in HPA-axis regulation is reported for two single nucleotide polymorphisms. One of these SNPs, designated MRI180V or rs5522 is located in exon 2. The SNP results in an isoleucine to valine amino acid change in the N-terminal domain of the protein and influences the transactivation capacity of the MR *in vitro* (DeRijk et al., 2006; Arai et al., 2003). MR180V is associated with enhanced cortisol, and autonomic responses to an acute psychosocial stressor measured with the TSST in young male twins (DeRijk et al., 2006). However, the enhanced cortisol response to the TSST was not found in a smaller study; instead an association between MR180V and higher ACTH responses and anxiety was observed during the

second TSST performed in this group (Ising et al., 2008). In addition, more feelings of depression have been observed in a cohort consisting of very old predominantly female individuals (Kuningas et al., 2007).

A second SNP tested in the MR is MR-2G/C or rs2070951, located two nucleotides before the first translational startsite of the MR. The C allele of the SNP creates a stronger translation site resulting in more MR protein expression compared to the G allele (this thesis chapter 3). An *in vitro* study demonstrated a change in transactivational capacity (van Leeuwen et al., 2010; Arai et al., 2003). Furthermore, the C variant of MR-2G/C is associated with lower basal cortisol levels in an elderly cohort (Kuningas et al., 2007).

In this study we aim to show involvement of the MR gene variants in neuroticism and mood changes in both controls and patients. Therefore we tested SNPs and haplotypes in the MR for associations with personality, mood and anxiety disorders in a cohort containing healthy controls and patients.

Materials and Methods

Subjects

Hundred patients (mean age 33.1 SD 11.2 years, 65 females) with a depressive and/or anxiety disorder were recruited from the outpatient department of the mental health center Rivierduinen in Leiden, the Netherlands. Fifty healthy controls (mean age 35.7 SD 14.0, 47 females) were recruited by advertisement. History of neurological or endocrine diseases, serious medical conditions, substance or alcohol abuse, pregnancy ovariectomy, psychotropic medication except a low dose of a benzodiazepine (equivalent to 30 mg oxazepam daily) and corticosteroid, estrogen, thyroid hormone, or herbal medication use were exclusion criteria. All subjects were subjected to a routine physical examination. Written informed consent was obtained from all participants. The study was approved by the ethics committee of the Leiden University Medical Center.

Psychopathological and psychological measures

All patients were diagnosed with the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Diagnoses of the patients and absence op psychiatric illnesses in healthy controls was confirmed by trained interviewers using the Dutch version of the Mini International Neuropsychiatric Interview Plus 5.0.0-R (van Vliet and de Beurs, 2007; Sheehan et al., 1998).

All subjects completed the Dutch translation of the Brief Symptom Inventory (BSI) and the mood and axiety symptom questionnaire (MASQ). The BSI is a shortened version of the Symptom Checklist (SCL-90), that is used to measure psychological complaints or symptoms (de Beurs et al., 2007). The total BSI-score generates an overall measure of psychopathological symptom severity. Internal consistency of the BSI is very good (Cronbach's $\alpha = 0.96$), and validity is sufficient (de Beurs et al., 2007). The MASQ measures three scales of General Distress: depressive symptoms (12 items), anxious symptoms (11 items) and mixed symptoms (15 items), it has a anxiety-specific scale (Anxious Arousal, 17 items) and depression-specific scale (Anhedonic Depression, 22 items). The reported internal consistency for each scale is excellent with coefficient alphas ranging from

0.78 to 0.92 (Buckby et al., 2007). For both BSI and MASQ (sub)scales, higher scores reflect more symptoms.
Neuroticism was assessed with the neuroticism subscale of the NEO Five Factor Inventory (NEO-FFI)(Costa and McCrae, 1992).

Genotyping
DNA was extracted from EDTA blood samples using a QIAgen DNA blood maxi kit according to the manufacturer's protocol (QIAgen, Venlo, the Netherlands). All subjects were genotyped for rs2070951, rs5522, rs5525, rs5534, rs6535578 and rs6535579 with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc., San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions and 10% of the samples were measured in duplicate. With this method it was not possible to measure rs2871 therefore SNPs rs65365578 and rs6535579 were measured instead since there is high LD between these SNPs and rs2871 accoring to the HAPMAP database. In addition, SNPs MR-2G/C and MRI180V were also genotyped using the TaqMan pre-designed SNP genotyping assays, assay ID C12007869_20 and C1594392_10 respectively, in combination with TaqMan universal PCR master mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Statistical analysis
Haploview (Barrett et al., 2005b) was used to calculate Hardy Weinberg equilibrium (HWE) and linkage disequilibrium between the SNPs (estimated with D' and r^2). Haplotypes were estimated and assigned to each individual using SNPHAP.

All other statistical analysis was performed with SPSS 16.0 (Chicago, IL, USA). To test if the MR genotype and haplotype distribution is equal between the patient and control group X^2 tests were computed. One-way ANOVA was used to compare BSI and neuroticism scores between patients and controls and regression analysis was carried out to analyze the effects of MR haplotypes on BSI or neuroticism scores.

Results

MR genotypes and haplotypes
All samples were genotyped for the three SNPs in the coding region, rs2070951, rs5522 and rs5525 and three intronic SNPs rs2070950, rs6535578 and rs6535579 using the Sequenom MassARRAY methodology (Table 1). The exonic SNP rs2871 could not be measured therefore the two intronic SNPs rs6535578 and rs6535579 were measured instead. The two previously reported SNPs MR-2G/C and MRI180V were additionally genotyped with a Taqman genotyping assay yielding identical results. All genotypes were in Hardy Weinberg equilibrium (data not shown). LD and haplotype analyses revealed two haplotype blocks. One, containing the SNPs in the first intron and exon 2. The other, containing SNPs in exon 9 and the preceding intron (Fig. 1a and b).

Table 1. Genotype frequencies (in %) in patients and controls and the statistical comparison of genotypes between patients (n=100) and controls (n=50)

SNP	variation	homozygotes		heterozygotes		homozygotes		χ-square
		patients	controls	patients	controls	patients	controls	p-value
rs2070950	C/G	29.4	26.0	42.2	54.2	28.4	20.0	0.352
rs2070951	G/C	29.4	26.0	42.2	54.2	28.4	20.0	0.352
rs5522	G/A	77.5	78.0	18.6	22.0	3.9	0.0	0.340
rs5525	T/C	77.5	78.0	18.6	22.0	3.9	0.0	0.340
rs5534	G/A	31.4	28.0	51.9	54.0	15.7	18.0	0.886
rs6535578	A/C	46.1	38.0	42.2	46.0	12.7	14.0	0.429
rs6535579	A/G	10.8	12.0	40.2	46.0	48.0	40.0	0.506

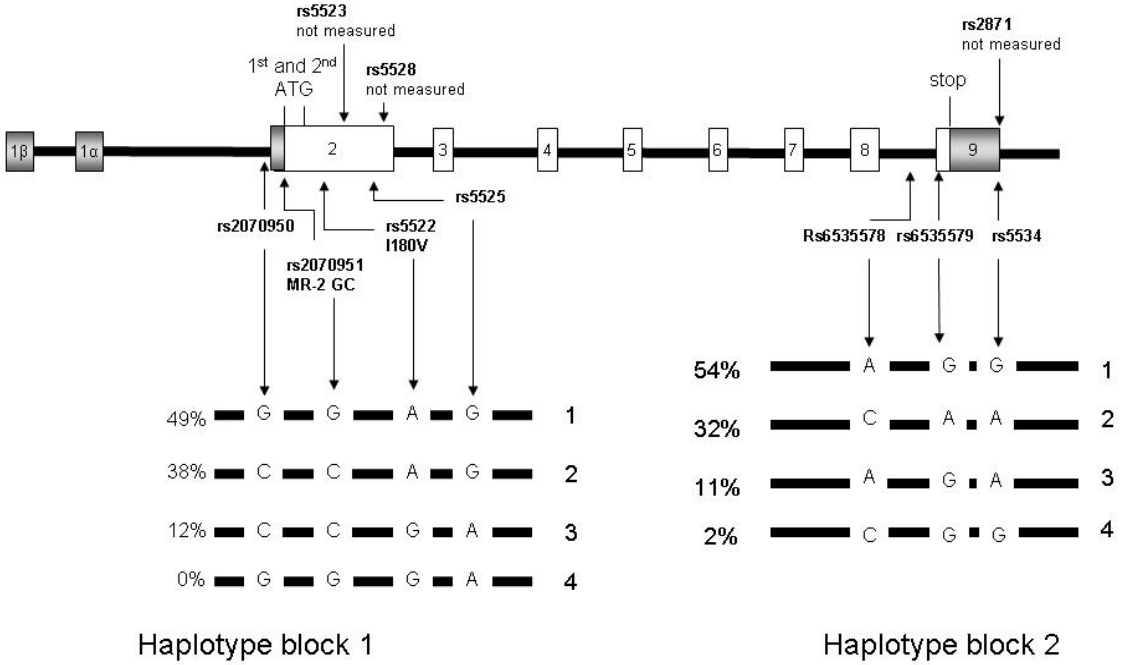

Fig. 1a. haplotypes in the MR

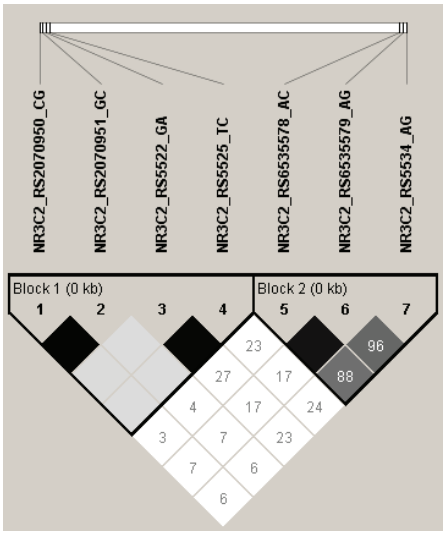

Fig. 1b. LD between MR SNPs measured in r^2 Black squares indicate r^2= 1 and shades of grey indicate r^2<1

Psychopathology

The genotype distribution of the seven different MR SNPs was not significantly different between the patient group and the control group (Table 2). Also the haplotype frequencies were not significantly different between the patient two groups (χ^2= 5.0 p=0.167; Table 3).

Table 3. Haplotype frequencies in patients (n=100) and controls (n=50)

	Haplotype Block 1			Haplotype Bock 2			
	1	2	3	1	2	3	4
Patients	45.9	42.9	11.2	54.8	30.1	12.0	2.0
Controls	50.0	36.7	13.4	53.1	36.7	9.2	1.0

The total score on the Brief Symptom Inventory (BSI) was significantly higher in the patients compared to the controls (patients 1.31 ±0.69 versus controls 0.18 ±0.18) and also on the eight different subscales the patients scored significantly higher (data not shown). There were no significant associations between the haplotypes and BSI total scores or subscale scores (all p>0.05), although there was a trend towards significance ($F_{1,98}$=3.34 p=0.071) that patients carrying the haplotype 2 of block 1 had a higher score on the anxiety subscale of the BSI (carriers 2.17 ±0.54 versus non-carriers 1.29 ±0.096). Also general distress, anhedonic depression, anxious arousal measured with the the mood and anxiety symptom questionnaire (MASQ) were not associated with MR haplotypes or single SNPs (all p>0.05)

Neuroticism

Neuroticism scores were significantly higher in the patient group compared to the control group (patients 44.1±7.9 versus controls 27.4±7.0) therefore both groups were analyzed separately. In the patients there was a significant association between both MR haplotype blocks and neuroticism; patients carrying one copy of allele 3 of haplotype block 1 (MR 180V carriers) had lower scores than non carriers and the scores were even lower in the patients carrying two copies of allele 3 (p= 0.04; Table. 4.). In block 2 homozygote carriers of allele 2 had lower scores of neuroticism compared to patients without allele 2 or only one copy of allele 2 (p= 0.02; Table 4.). There was no effect of the MR haplotypes on neuroticism scores in healthy individuals (p>0.05).

Table 4. Neuroticism scores in patients carrying 0, 1 or 2 copies of a haplotype

Haplotype	**Number of copies**			
Block 1	0	1	3	p
1	44.1 (±7.9)	43.6 (±8.4)	45.0 (±7.2)	n.s.
2	42. 7 (±8.0)	44.7 (±7.8)	46.3 (±7.3)	n.s.
3	45.3 (±7.4)	41.1 (±8.5)	37.3 (±7.1)	0.02
Block 2				
1	38.6 (±8.9)	45.5 (±7.6)	44.8 (±6.5)	n.s.
2	44.5 (±7.0)	45.4 (±7.3)	35.1 (±10.1)	0.02
3	44.1 (±8.1)	44.0 (±7.2)		n.s

Discussion

We described two haplotype blocks in the human MR-gene, which were associated with neuroticism but only in depressed and/or anxiety patients. However, the SNPs and the haplotype blocks in the MR gene were not associated with mood and/or anxiety disorders.

Neuroticism

Neuroticism was associated with both haplotype block 1 and 2 in the patient group; this is the first study showing that genetic variation in the MR is associated with neuroticism. Previous studies demonstrated heritability of neuroticism, associations between cortisol and neuroticism, involvement of the MR in behavior and a role for genetic variation in stress responsiveness reviewed by (Flint, 2004). The effect of the MR was only observed in the patient group, which as a total had significantly higher neuroticism scores. A depressive state amplifies the personality profile and the scores can be interpreted as an accurate reflection of the current condition of the person (Costa, Jr. et al., 2005). Therefore it is likely that the observed effect is only visible in this group with amplified scores. Another explanation why the effect is not observed in the control group might be the relative small group size (n=50) as compared to the patient group (n=100). However, also in another cohort consisting of healthy individuals from the German population we also did not observe an association between the current described MR haplotypes and neuroticism (data not shown).

Mood and anxiety

In this study we did not find an association between MR SNPs and haplotypes with mood and/or anxiety disorders. A previous study did report an association between the MR180V SNP and feelings of depression in a cohort consisting of healthy elderly (Kuningas et al., 2007). The large difference in age between the two groups might explain the discrepancy between the two findings since it is known from animal studies that MR expression decreases with aging and we demonstrated that MR haplotypes influence MR functionality and expression *in vitro* (van Leeuwen et al., 2010). It is possible that the effect of the MR haplotypes is only observed in elderly were the MR expression is probably already low. The relatively small group sizes, the patient group consisted of hundred individuals and the control group consisted of fifty individuals, might also be a reason why we did not find an association.

Mechanism

Although the mechanism of the SNPs in block 1 is partly known from previous *in vitro* experiments, it is difficult to explain the *in vivo* consequences based on these *in vitro* changes. In block 1 haplotype 3 is the allele associated with neuroticism, this is the only allele in our cohort containing the minor allele of MRI180V which leads to a lower transcriptional efficiency, without influencing the MR expression. The C allele of MR-2G/C is present in both haplotype 2 and 3 and is increasing the transactivational capacity by increasing the MR protein expression compared to the G allele of MR-2G/C present in haplotype 1(van Leeuwen et al., 2010). It seems that in block 1 MRI180V or the *in vitro* not tested SNP rs5525 causes the effect on neurotisicm. How this effect is mediated needs further investigation since both haplotypes 1 and 3 affect transactivation. In haplotype 1 this is mediated by the C allele of MR-2G/C, in haplotype 3 it is mediated by the G

allele of MRI180V while only haplotype 3 is associated with neurotiscism. Interestingly haplotype 4 which should contain both alleles that result in the lower transactivation, the G allele MR-2G/C and the G allele of MRI180V, is not observed in our population. This might occur by chance but one can speculate that this combination has negative consequences for a person's health and is therefore not passed on from generation to generation in the population.

In block 2, allele 2 is the allele associated with neuroticism. The SNPs in this block are not yet tested for functionality but computer screenings revealed that one of the SNPs rs2781 promotes loop formation of the mRNA and this will probably alter the mRNA stability. By influencing mRNA stability SNPs can increase or decrease expression of MR protein, if the mRNA is present for a longer period more protein can be formed and subsequently, more protein will lead to a higher transcriptional activity.

Furthermore, although there are no SNPs in the coding regions in the two described haplotypes blocks there are many SNPs in the intronic regions between the two blocks (described in more detail in chapter 7, the general discussion of this thesis). The intronic SNPs might affect the MR by influencing RNA splicing or transcription of the MR by changing binding of cofactors. We can not rule out that a SNP in such region is causing the effect on neuroticism.
In conclusion, there are two main haplotype blocks in the MR, these blocks were not associated with mood and/or anxiety disorders but there was an association with neuroticism in the patient group indicating involvement of the MR in human behavior.

7

General Discussion

Contents

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA)-axis, resulting in production and secretion of corticosteroids which facilitate physiological and behavioural adaptation. These effects exerted by corticosteroids are mediated by two brain corticosteroid receptor types, the mineralocorticoid receptor (MR), with a high affinity already occupied under basal conditions and the glucocorticoid receptor (GR), with a low affinity only activated during stress. The HPA axis response to stressors is highly variable between individuals. These individual differences are partly inherited and probably underlie the individual's vulnerability or resilience to stress-related psychopathology. The MR is at least involved in two important systems in the human body, the HPA axis and the renin-angiotensin-aldosterone-system while animal studies demonstrated that a complete knock-out of the MR is not compatible with life.

This project was designed to identify and characterize genetic variation in the MR and to test the effect of the genetic variation on the stress response in healthy individuals and the role of this in psychopathology. In addition, effects of genetic variation in MR on the renin-angiotensin-aldosterone system were investigated.

1. Location of genetic variation in the MR

Single Nucleotide Polymorphisms (SNPs) are common variations at the DNA level; by definition the frequency of SNPs in the normal population is more than 1%. In the MR, SNPs are located in the transactivation domain, the intronic regions and the untranslated exonic regions. There are no SNPs found in the DNA and the ligand binding domain of the MR. In contrast, mutations, genetic variation with a frequency of less then 1% in the population, are predominantly located in the DNA and ligand binding domain (previously reported and in this thesis described SNPs and mutations are shown in figure 1).

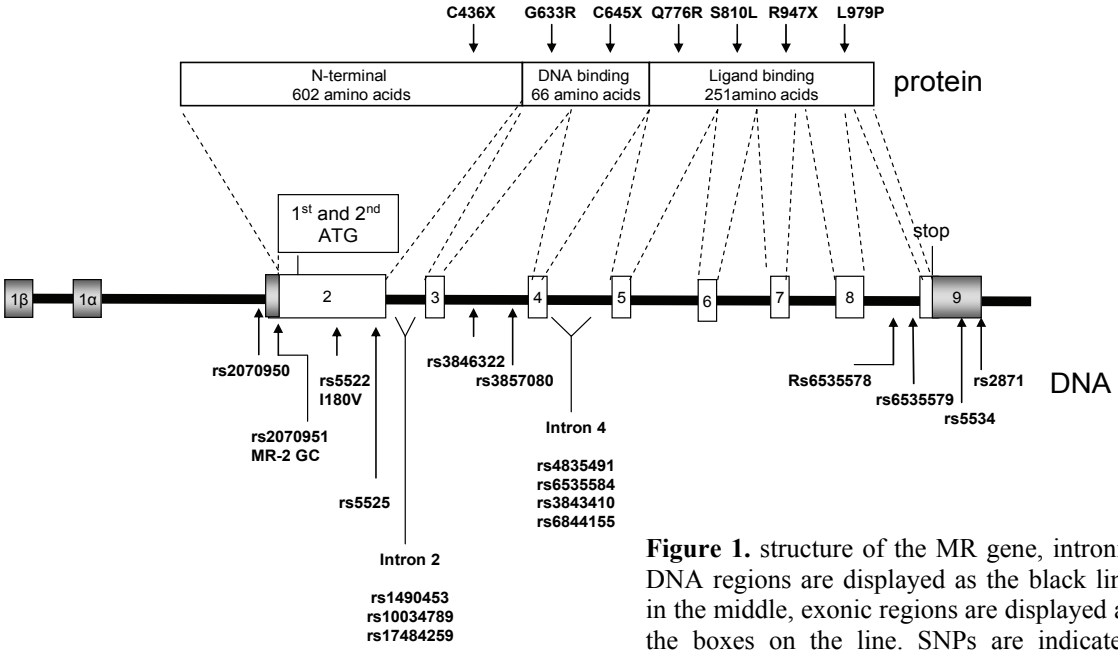

Figure 1. structure of the MR gene, intronic DNA regions are displayed as the black line in the middle, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA, while the protein structure and mutations are displayed above

The differential distribution of SNPs and mutations in the MR can be explained by their importance for the functioning of the receptor. Mutations in the DNA or ligand binding domains of the MR can lead to pseudohypo-aldosteronism type I (PHA1). Individuals with PHA1 have severe problems in maintaining electrolyte balance and untreated this will lead to death. In the genome there is a SNP approximately every 200 nucleotides. However, in the DNA and ligand binding domain of the MR, consisting of 950 nucleotides (this thesis, chapter 1), no SNPs have been observed. This is a second example demonstrating that genetic variation in those regions is probably too severe to be spread throughout the population.

Although SNPs in the MR have more subtle effects compared to mutations, their impact on general health might be considerable because of their high frequency in the population.

2. Haplotypes in the MR

In the human genome some regions show little historical recombination and subsequently within these regions only a few SNP combinations are observed. These SNP combinations are called haplotypes and the regions containing those haplotypes are called haplotype blocks. The boundaries of blocks and the specific haplotypes they contain are highly correlated across populations and are formed by recombination hotspots, sequences in the DNA where recombination occurs frequently.

The fact that two SNPs on nearby sites occur more often together due to the lack of recombination between the SNPs is called linkage disequilibrium and is measured in D' or r2. Both measures range from 0 (SNPs do not occur more frequently together) to 1. D' is defined in such a way that it is equal to 1 if just two or three of the four possible haplotypes are present and is <1 if all four possible haplotypes are present. The measure r2 is the statistical correlation between the two SNPs and a value of 1 means that only 2 of the four possible haplotypes are present. Intermediate values of the measures are difficult to interpret and to compare.

The existence of haplotypes and linkage disequilibrium is an important factor in genetic studies because when an association is found between a SNP and a phenotype it does not necessarily mean that there is a causal relationship. The SNP can be in linkage disequilibrium with the causal SNP that is located in the same gene or even in an adjacent gene. Thus when studying the mechanism and physiological function of genetic variability, it is crucial to take haplotypes into consideration.

Measuring SNPs in the promoter and exonic regions of the MR gene revealed two haplotype blocks (Fig 2 and chapter 5 of this thesis).

However, analyzing the complete MR gene including the intronic region in data from the HAPMAP consortium, an organization with the goal developing a haplotype map of the human genome, results in more haplotype blocks, the two blocks we identified but also blocks in between those two blocks containing intronic SNPs.

Linkage disequilibrium between the SNPs described in this thesis, located in and before exon 2 (5' of the gene) and in and just before exon 9 (3' of the gene) and previously reported SNPs located in intron 2, 3 and 4 was calculated in the HAPMAP data (Fig 3).

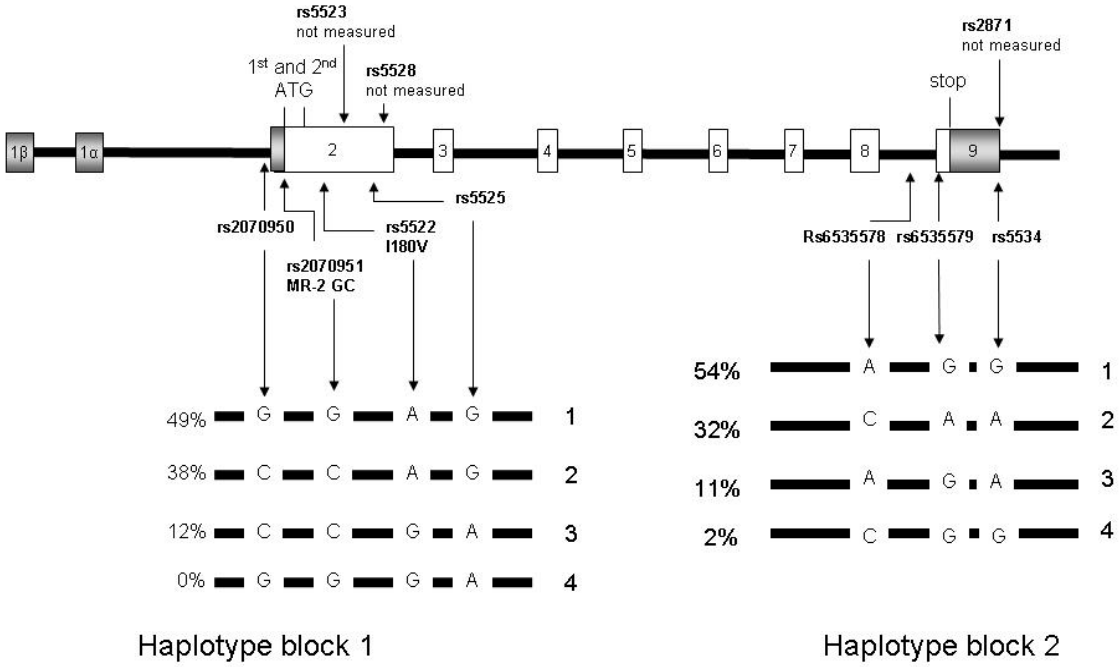

Figure 2. The hMR gene with SNPs and the two haplotype blocks formed by these SNPs. Intronic DNA regions are displayed as the black line on top, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA and the haplotypes formed by the SNPs are indicated under the SNPs.

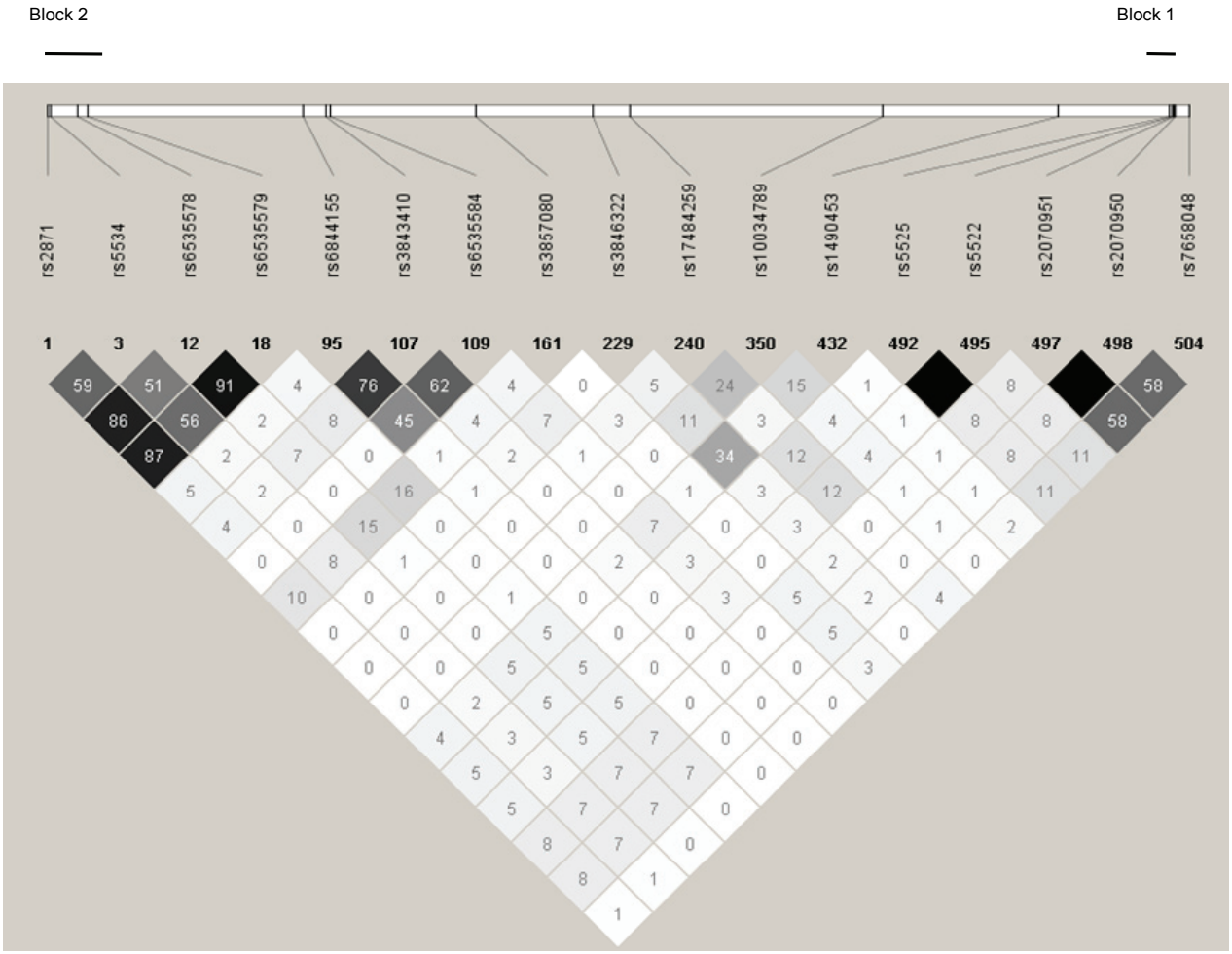

Figure 3. Linkage disequilibrium between the MR SNPs previously reported and reported in this manuscript. Black squares indicate $r^2= 1$ and shades of grey indicate $r^2<1$

The SNPs reported in this thesis, captured in haplotype block 1 and 2 appeared to be unrelated to the previously reported SNPs. Therefore the associations between MR SNPs and blood pressure previously found (Zemunik et al., 2009; Tobin et al., 2008) are probably not mediated by the SNPs described in this thesis and vice versa the associations described in this thesis are not mediated by the intronic SNPs

3. Predicting functionality of SNPs and haplotypes

Putative functionality of a SNP can be predicted based on the location. For example, an amino acid changing SNP is more likely to be functional than a synonymous coding SNP. Other putative functional SNPs include SNPs located in regulatory regions, regions involved in mRNA stability, RNA splicing regions and micro RNA binding sites. Web based computer programs which detect those specific regions can help in predicting the functionality of the SNPs. The different computer programs are listed in table 1.

Table 1. Computer programs used for predicting functionality of SNPs

Sequence detected	program	website
Amino acid change		http://www.ncbi.nlm.nih.gov/snp/
Transcription factor binding site	TFsearch (Heinemeyer 1998)	http://www.rwcp.or.jp/papia/
Splice site		http://www.fruitfly.org/seq_tools/splice.html
mRNA stability	Mfold (Zuker, 2003)	http://mfold.bioinfo.rpi.edu/
Micro RNA binding site	Target scan	http://www.targetscan.org/

Four previous studies report associations with MR SNPs but only two studies specifically tested the putative functional SNPs. Table 2 summarizes MR SNPs and their predicted effect based on location predicted by the different computer programs.

Table 2. Putative function of SNPs in the MR

SNP	variation	Location	Putative function	literature
rs7671250	T/C		No transcription factor site	Chapter 5 this thesis
rs6814934	C/G		Possible TATA, Th1E4, ETS site	Chapter 5 this thesis
rs7658048	C/T		T→ possible GATA-2 site	Chapter 5 this thesis
rs2070950	C/G		No transcription factor site	Chapter 5 this thesis
rs2070951	G/C	Exon 2 5'UTR	In Kozak translation regulatory region	Van Leeuwen 2010, this thesis
rs5522	G/A	Exon 2	Amino acid change	DeRijk 2006
rs5525	T/C	Exon 2	No aminoacid change	Chapter 5 this thesis
rs1490453		Intron 2	No effect on splicing	Zemunik
rs10034789	G/A	Intron 2	No effect on splicing	Tobin 2008
rs17484259	T/G	Intron 2	No effect on splicing	Tobin 2008
rs3846322	C/T	Intron 3	No effect on splicing	Tobin 2008
rs3857080	G/C	Intron 3	No effect on splicing	Tobin 2008
rs4835491	C/T	Intron 4	No effect on splicing	Tobin 2008
rs6535584	T/C	Intron 4	No effect on splicing	Tobin 2008
rs3843410	C/A	Intron 4	No effect on splicing	Tobin 2008
rs6844155	C/T	Intron 4	No effect on splicing	Tobin 2008
rs5534	A/G	Exon 9	No aminoacid change	Chapter 5 this thesis
rs2871	T/C	Exon 9 3'UTR	Possible loop formation mRNA Close to miRNA binding site	Chapter 5 this thesis

4. Testing SNPs and haplotypes *in vitro*

In the classical view the MR functions as a transcription factor, when ligand binds to the MR in the cytoplasm, the MR dimerizes with either GR or MR and translocates to the nucleus. In the nucleus the MR binds to specific DNA sequences and influences transcription of specific genes. Different aspects in this process can be influenced by genetic variation. The two SNPs MR-2G/C and MR180V were extensively studied for their functionality *in vitro* (table 3).

Table 3. *In vitro* effects MR-2G/C and MRI180V

SNP	mRNA	protein	Ligand binding Kd	Ligand binding Bmax	Transactivation	Literature
-2G/C	No effect	C → ↑ MR protein	No effect	C → ↑ B max	C → ↑ transactivation with cort, aldo and dex C → ↓ transactivation with aldo	This thesis Arai 2003
I180V	No effect	No effect	No effect	No effect	V → ↓ transactivation with cort and dex V → ↓ transactivation with cort no effect with aldo V → ↓ transactivation with aldo	This thesis DeRijk 2006 Arai 2003

4.1. MR-2G/C

As predicted based on the location in the first Kozak region of the MR, the C allele of MR-2G/C results in more MR protein due to increased translation. The mRNA was not affected by the SNP therefore it is only the translation that is influenced and not the mRNA synthesis. Although there are two translation start sites in the MR resulting in respectively MR-A and MR-B, the COS-1 cells that were transfected with human MR only expressed MR-A. The existence of MR-B in other cell lines and *in vivo* remains to be elucidated. As far as we know, MR-B is only detected with *in vitro* translation, a method where the complete translation process is performed in a reaction tube. There are no studies showing MR-B expression *in vivo* or in transfected cells. The increased MR protein expression with C allele subsequently leads to the observed increase in transactivation and the increased binding capacity of the MR.

However, the increase in transactivation was not observed in all studies. In contrast Arai et al reported a decrease in transactivation with MR-2 C (Arai et al., 2003). The methodology between the studies differs on several points and this probably accounts for the inconsistency of the studies. The main discrepancy is the concentration used. In our study using similar cells, a wide concentration range starting at 10^{-13}M to 10^{-8}M was used and maximal transactivation was already observed at 10^{-10}M aldosterone (this thesis chapter 4 Fig. 2.a.). In the study by Arai et al the lowest concentrations used were 10^{-11}M and 10^{-10}M therefore it might be possible that the real maximal transactivation is not observed because the concentrations were too high. It is difficult to relate the concentrations used in vitro with concentrations observed in vivo because *in vivo* only 10-20% is unbound and biological active. In blood reference values for cortisol are 0,2 - 0,6 µmol/l in the morning and 0,1 - 0,4 µmol/l in the afternoon, of which 10-20% of this circulating cortisol is unbound and active. One study reported cortisol levels in cerebro spinal fluid (CSF) that were 16.5 nmol/l (Raubenheimer et al., 2006) and one study reported cortisol levels in post mortem brain, the levels were on average 0.65 µmol/l (Karssen et al., 2001)

4.2. MRI180V

MRI180V results in an isoleucine → valine amino acid change in the transactivational domain of the MR. DeRijk et al demonstrated a ligand dependent effect on transactivational capacity. MR180V leads to a lower transactivational capacity but this effect was only observed when cortisol was used as a ligand. The effect was not observed with aldosterone in this experimental set up. In contrast, Arai et al demonstrated significant differences in transactivation using aldosterone (Arai et al., 2003). As described in the previous paragraph for MR-2G/C differences in methodology might account for this.

The effect on transactivation of MRI180V was not mediated by differences in expression of the MR or ligand binding since those were not influenced by the SNP (this thesis chapter 4). However, it is known that co-factors influence binding of the ligands but we did not assess ligand binding in the presence of human co-factors. Therefore it is still possible that co-factor binding is influenced by the MRI180V resulting in the observed difference in the transactivation assay with cortisol and aldosterone, this mechanism remains to be elucidated.

Several other possible mechanisms of action of MRI180V are currently being investigated. Preliminary data indicate that the translocation of the MR 180V from the cytoplasm to the nucleus is less efficient as compared to the MR I180 (DeRijk, Weij and van Leeuwen, in preparation). In addition the transactivation of endogenous genes is currently being tested (deRijk). Furthermore, *in vivo* the MR dimerizes with either MR or GR. All in vitro experiments were performed in cell lines with no or very low endogenous MR or GR expression. The influence of MRI180V in combination with GR expression is not being investigated. It is possible that the dimerization with GR is influenced by MRI180V.

4.3. Haplotypes

Previous studies tested the MR SNPs separately; in this thesis we additionally tested the combinations of MR-2G/C and MRI180V. The combination of these two SNPs is not the complete haplotype 1; the promoter and intronic SNPs were not included in the test. Although both SNPs influence the MR there was no significant interaction between MR-2G/C and MRI180V in transactivation of the MR and MR protein expression (chapter 4). However, confirmation with other assays is needed to completely exclude interaction between the SNPs.

4.4. Conclusion testing SNPs and haplotypes *in vitro*

The two MR SNPs that were predicted to be functional based on computer screenings, MR-2G/C and MR I180V, were indeed functional in *in vitro* transactivation assays. MR-2G/C influences the translation and thereby protein expression with subsequent effects on transactivation and ligand binding capacity. MRI180V influenced transactivation but the underlying mechanism is not yet elucidated. In our assays there was no interaction between the SNPs. For further experiments we propose to use endogenous MR responsive genes as output measures for MR transactivational capacity instead of artificial systems such as the luciferase system. This will give more insight into the mechanism affected by the genetic variation. Furthermore we suggest including the GR and co-factors in the in vitro studies since MR dimerizes with either MR or GR. Genetic variation might

influence dimerization with the GR or co-factor binding. In addition, genetic variation in the GR or co-factors might influence the MR as well.

5. Association studies MR

5.1. Overview associations MR

Several studies, including the studies described in this thesis, reported associations with MR SNPs (table 4). The MR is involved in important systems in the body and this is reflected in the associations found. On the one hand, there are several associations with blood pressure. This is probably mediated by the MR in the renin-angiotensin- aldosterone system, the system regulating electrolyte and water balance. On the other hand, there are associations related to the regulation of the HPA axis, with the TSST responses and the CAR after dexamethasone administration. In addition, we described associations with MR SNPs and feelings of depression and neuroticism

Table 4. Associations with MR SNPs and MR haplotypes

SNPs	Haplotype block	Association	literature
	1	Hap 3→ neuroticism ↓	Chapter 5 this thesis
		No association psychopathology, Hap 2b → trend with ↑ anxiety (p=0.072)	
		Hap 2→ TSST responses ↑	Chapter 4 this thesis
rs7671250	1	Not tested separately	
rs6814934	1	Not tested separately	
rs7658048	1	Not tested separately	
rs2070950	1	Not tested separately	
rs2070951	1	GG→ cortisol awakening response after dexmethasone males ↑ females ↓	Van Leeuwen 2009
		G→ renin angiotensin aldosterone system and blood pressure ↑	Chapter 3 this thesis
		No association hypertension	Martinez 2009
		No association blood pressure	Tobin 2008
		C→ cortisol in the morning ↓	Kuningas 2007
rs5522	1	V→ cortisol and autonomic responses to the TSST ↑	DeRijk 2006
		V→ only ACTH and anxiety ↑ during the second TSST	Ising 2008
		V→ more feelings of depression	Kuningas 2007
		V→ protection hypertension	Martinez 2009
		V→ cortisol awakening response after dexamethasone ↓ in males in females no effect	Van Leeuwen 2009
		No association blood pressure	Tobin 2008
rs5525	1	Not tested separately	
rs1490453	Between 1 and 2	Fibrinogen levels	Zemunik
rs10034789	Between 1 and 2	Mean night DBP	Tobin 2008
rs17484259	Between 1 and 2	Clinic SBP	Tobin 2008
rs3846322	Between 1 and 2	Clinic SBP	Tobin 2008
rs3857080	Between 1 and 2	Mean night DBP Mean night SBP	Tobin 2008
rs4835491	Between 1 and 2	Clinic DBP	Tobin 2008
rs6535584	Between 1 and 2	Clinic DBP	Tobin 2008
rs3843410	Between 1 and 2	Clinic DBP	Tobin 2008
rs6844155	Between 1 and 2	Clinic DBP	Tobin 2008
	2	Hap 2→ neuroticism ↓	Chapter 5 this thesis
rs5534	2	Not tested separately	
rs2871	2	Not tested separately	

5.2. Associations related to the HPA axis and other brain regions

The HPA axis can be tested at different levels with different tests; these tests are described in detail in chapter 1, the introduction of this thesis. The two functional SNPs MR-2G/C and MRI180V have been tested for associations with the outcome measures of HPA axis tests.

Kuningas et al reported an association between MR-2G/C and morning cortisol levels in the elderly. However, we did not find an association with basal cortisol levels, the levels without the presence of a stressor and MR SNPs in our cohorts, consisting of younger individuals. Also Klok et al. did not find associations between the CAR and MR-SNPs (Klok and DeRijk, *in press* PNEC). Differences in age could account for the discrepancies between the studies. It is known from animal studies that MR expression alters with age and the associated MR-2G/C influences the expression (demonstrated with *in vitro* assays described in chapter 2 and 3 of this thesis). Therefore, it is possible that the impact of MR-2G/C changes with age.

Suppression of the HPA axis was tested with a low dose of dexamethasone. Oral administration of 0.25mg dexamethasone at 2300h resulted in a significant suppression of the cortisol awakening response (CAR). Both SNPs modulated the suppression of the CAR after dexamethasone significantly and in a sex-specific manner. There are several explanations how MR gene variation can influence dexamethasone-induced suppression of the CAR.

First of all, MR gene variants, might react differently *in vivo* to stimulation with dexamethasone, as we observed *in vitro*, and thus also differentially affect HPA axis suppression. This is a likely explanation, since without dexamethasone there was no genotype effect on the CAR and dexamethasone has an appreciable affinity for the MR (Grossmann et al., 2004; Rupprecht et al., 1993). However, central brain MR is poorly accessible for dexamethasone since Pg-p hampers the penetration of the steroid into the brain (Meijer et al., 1998). In spite of this, some dexamethasone will pass the blood brain barrier and activate MR, while the pituitary gland also contains some MR.

Secondly, 0.25mg dexamethasone results in lower, but still appreciable levels of saliva cortisol (Fig. 3). The levels are comparable to the levels normally observed in the afternoon and are probably sufficient to activate MR (Droste et al., 2008; Karssen et al., 2005; Meijer et al., 1998) It is hypothesized that under these conditions the differential effects of the MR gene variants appear with subsequent effects on the input from higher limbic brain regions on the hypothalamic AVP (and/or CRH) drive to pituitary ACTH release (Kovacs et al., 2000; Tajima et al., 1999; Bradbury et al., 1994) Since limbic MR has inhibitory effects on the HPA axis, a reduced MR activation may result in this enhanced drive (Holsboer, 2000).

Finally, fast non-genomic actions of membrane bound MR might be involved. Membrane bound MR has much lower affinity for corticosterone then the classic MR with its genomic actions (Joels et al., 2008; Karst and Joels, 2005) and was recently shown in rats to mediate fast feedback during the ultradian pulse (Atkinson et al., 2008). These membrane bound MR have low affinity for corticosteroids and require rising corticosteroid levels for activation which makes a role for this non-genomic mechanism less likely. The possible mechanisms are described in more detail in chapter 2 of this thesis and in section 5.4 of this discussion the gender issue will be discussed.

Activation of the HPA axis was tested with a psychosocial stressor, the TSST. DeRijk et al demonstrated that MR180V enhanced cortisol and heart rate responses to the psychosocial stressor; MR-2G/C was not tested in this cohort. Ising et al did report an association between MR180V and increased ACTH levels and anxiety but only during the second TSST (Ising et al., 2008). In the study described in chapter 4 of this thesis, carriers of 2 copies of Hap 2 from haplotype block 1, that is the haplotype containing MR-2C and MRI180, showed higher plasma and saliva cortisol, ACTH levels and heart rate. These three studies clearly demonstrate that the two MR SNPs influence the response to a psychosocial stressor. The precise mechanism how haplotoype 2, which shows an increased MR expression and transactivation capacity *in vitro*, leads to more reactive HPA axis responses and resilient behavior to stressors is unknown. MR-expression is essential for neuronal protection and stability of neuronal circuits (Lai et al., 2009; de Kloet et al., 2007). The low affinity membrane form of the MR becomes activated during stress levels of cortisol and increases excitatory glutaminergic transmission while decreasing post-synaptic after-hyperpolarization (Joels et al., 2008). This rapid excitatory MR-mediated effect may very well underlie the non-genomic actions exerted by cortisol on neuroendocrine, emotional and cognitive processes (Brinks et al., 2009). Therefore, it will be a challenge for future research to dissociate during a psychosocial stressor the genomic and non-genomic effects mediated by the MR on the processing of stressful information resulting in HPA axis reactivity and behavior. The MR haplotypes identified in this study may be very helpful in this respect.

Since MR-2G/C and MRI180V were both functional *in vitro* and influenced suppression and activation of the HPA axis effects on psychopathology were expected. The results, however, were inconclusive. Kuningas reported more feelings of depression in carriers of MRI180V in a healthy elderly cohort but we were not able to find an association between the SNPs and mood and anxiety disorders in a cohort containing one hundred patients with mood and/or anxiety disorders and fifty healthy individuals (this thesis chapter 5). The small group size in our study is a clear limitation since the allele frequency of MRI180V is low, it is only 10%. Although we did not find an association with psychopathology there was an association with neuroticism, a personality trait that is regarded as an endophenotype for depression. Future studies using larger cohorts and including not only the phenotype depression and endophenotype neuroticism but also environmental factors and biological markers are needed for elucidation of the consequences of the MR SNPs.

5.3. Associations related to the Renin Angiotensin system

MR-2G/C influences the expression of the MR and this not only affects cortisol mediated transactivation, also aldosterone mediated transactivation is altered by the SNP (*in vitro* assays chapter 4 this thesis). In three different cohorts and with different tests we showed effects of MR-2G/C on the Renin Angiotensin system (RAS) (chapter 4 this thesis). Individuals carrying the GG genotype had higher blood pressure and plasma renin levels as compared to individuals with the CC genotype (more details chapter 4 of this thesis). Martinez et al did not report an association with hypertension and MR-2G/C but the method of analysis differs between the studies and this might explain the discrepancy (Martinez et al., 2009). Martinez et al created haplotypes with SNPs from different haplotype blocks and this results in small groups. They reported that the haplotype

containing the G allele of MRI180V was associated with a reduced risk of hypertension and concluded that MRI180V G was associated with a reduced risk of hypertension. However, in all our studies the MRI180V G allele is only observed together with the MR-2G/C C allele therefore it is still possible that MR-2G/C is causing the effect in the Martinez study as well but remains unnoticed due to the large amount of haplotypes containing the C allele of MR-2G/C. In our studies we tested MR-2G/C and MRI180V separately and only MR-2G/C showed the effect.

Previously deRijk et al showed that MRI180V had no effect on transactivation of the MR *in vitro* when aldosterone was used as a ligand. This is reflected in an association study where they showed that MRI180V was not associated with different aspects of the renin angiotensin aldosterone system; there was no effect of the SNP on blood pressure, renin and aldosterone secretion or Weinbergers salt loading test.

Martinez et all showed an association with MRI180V and risk for hypertension but as explained before, the analysis is different and this might explain the discrepancy.

In addition, Tobin et al tested 88 SNPs in the MR and showed that 8 SNPs in intronic regions were associated with blood pressure. The putative function of the SNPs tested is unclear since computer screenings predicted no effects on splicing (table 2). It is possible that these SNPs are linked to functional variants and that these intronic SNPs are not causing the effect. However, in their study there was no effect of MR-2G/C and MRI180V on blood pressure so those are not the causal SNPs in this study.

Considering the different studies it can be concluded that SNPs in the MR influence salt regulation, blood pressure regulation and the development of hypertension. However, the causal SNPs in the MR are not yet elucidated. Based on predicted function, *in vitro* effects and our association study MR-2G/C is the most likely effect causing SNP but this was not observed in all studies. It is possible that other, currently untested SNPs, or combinations of SNPs cause the effect.

5.4. Effect of gender

Several associations with MR SNPs were gender dependent. For some associations males with a specific genotype had the complete opposite association compared to females. Animal studies demonstrated that sex hormones influence MR expression e.g. in rats, estrogens decrease the expression of the MR in the pituitary (Turner et al., 1990) and progesterone treatment increased the activity of the MR promoter in cell lines (Castren et al., 1995). The MR-2G/C SNP also influences the MR expression in vitro and it can be hypothesized that this interacts with the effects of the sex hormones. In addition, progesterone can bind MR, acting both as an agonist or antagonist. Furthermore oral contraceptives bind the MR and this leads to increased total cortisol levels while the free cortisol levels are unchanged. Interference of sex hormones was not tested in the *in vitro* assays performed; this should be included in future experiments.

5.5. Interaction with other genes

Complex diseases, like depression, are not caused by one single gene; several genes in combination with environmental factors are involved in the etiology. For optimal HPA axis regulation the

balance between MR and GR is important. Furthermore, GR SNPs are associated with the same HPA axis measures as the MR SNPs e.g. TSST response and cortisol after dexamethasone (see introduction). Therefore the influence of genetic variation in the GR in combination with genetic variation in the MR should be tested *in vitro* and *in vivo*. In the cohorts tested so far, interactions between MR and GR SNPs could not be tested due to the limited group sizes.

5.6. Associations MR SNPs with unknown function

Computer screenings revealed possible functionality of the SNPs in exon 9 that form haplotype block 2, therefore they were included in the association study. The SNPs were associated with neuroticism, thus *in vitro* functionality assays should now be performed to elucidate the underlying mechanism. Furthermore, the region between haplotype block 1 and 2 should be screened for SNPs and the functionality tested *in vitro*. There are associations found with SNPs in this region but it is unclear what the mechanism is. It is also unclear if the SNPs tested are the functional SNPs or that they are in linkage disequilibrium (LD) with the functional SNPs.

5.7. Predicting *in vivo* consequences based on *in vitro* studies

The studies in this thesis showed that it is difficult to predict the exact consequences of a SNP in the human body based on the functionality of the SNP in the *in vitro* assays. For example, most associations were gender dependent but interference of sex hormones was not tested in the *in vitro* assays performed. To obtain a better prediction, sex hormones should be included in the in vitro studies e.g. binding of the sex hormones might be influenced by the MR gene variants. This can be tested with the ligand binding assays we performed for assessing the impact of the MR gene variants on cortisol binding.

Furthermore, in the *in vitro* studies described in chapter 5, the effects of the combination of the two MR SNPs MR-2G/C and MRI180V were tested but there are many other SNPs in the MR (shown in Fig 1). Interactions of those SNPs were not tested *in vitro* and also other genes are not taken into account in the *in vitro* assays.

It will be difficult to predict the exact consequence *in vivo* based on functionality *in vitro* but the chance that a SNP is effective *in vivo* is higher when it is functional *in vitro* e.g. the two functional SNPs MR-2G/C and MRI180V and the putative functional SNPs in exon 9 showed effects *in vivo*.

6. Future perspectives

Our studies showed genetic variation in the MR, which appeared *in vitro* functional in translation, transactivation and binding properties of this receptor. Based on the findings in the association studies, we postulate that the genetic variants may modulate the stress response and affect in part a person's personality structure. Furthermore, the MR variants seem to influence the regulation of blood pressure and salt homeostasis.

Hence, one may wonder whether the MR genotype is of relevance for the prediction of either vulnerability or resilience to psychopathology. After all, MR mediates the genomic action of cortisol, a hormone that communicates stressful environmental influence to brain and body. This

implies that processing of environmental information may be genetically modified via central processes linked to the MR variants. At a behavioural level, these MR mediated central processes involve appraisal of stressful information and the regulation of the initial stress reaction.

Evidence from this thesis and other studies suggests that increased MR-expression is beneficial and neuroprotective. This notion is reinforced by the study of Otte et al., who recently showed accelerated responses to anti-depressive therapy by using the MR agonist fludrocortisone as add on. This finding is in line with our indications that the MR-2C variant, resulting in increased synthesis of MR protein, has beneficial effects. Moreover, the MRI180V variant has a remarkable ligand dependent enhancing effect on transactivation *in vitro*; the SNP influences transactivation mediated by cortisol, while there is no effect on transactivation when aldosterone was used as a ligand. This finding calls for studies to explore the steroid specificity of the MR variants.

The studies described in this thesis mainly focus on the MR. However, for full understanding of cortisol action in the brain, the balance between GR and MR needs to be taken into account.
Antidepressants influence both MR and GR expression and the GR antagonist mifepristone has been proposed as medication in severe depression. Moreover, genetic variation in the GR has been reported to influence the pathogenesis of depression and the efficacy of tricylcic anti-dpressants. An important question for future research is therefore how to reinstate imbalanced GR and MR mediated actions as a therapeutic strategy for stress-related disorders such as depression, taking their recently discovered genetic variation into account.

Summary

A depressive disorder is a common mood disorder characterized by loss of pleasure and a negative mood. Many people occasionally feel "depressed" but only if these feelings persist for a longer time it is possible that a person is suffering from depression. A major depression is a serious and common disease. It interferes with a person's ability to work, study, sleep, eat and enjoy pleasurable activities. Over 15% of the Dutch population experiences depression during their life. Although many studies were performed to elucidate the etiology of the disease, the mechanism has still not been completely clarified. Stress plays an important role in the etiology of depression and some families are more vulnerable, indicating a genetic component. Twin studies in which 100% identical monozygotic twins were compared with dizygotic twins, who are on average 50% identical, confirm that there is a genetic component involved in the development of a depression.

Depression is often associated with disturbances in the hypothalamus-pituitary-adrenal (HPA) axis. The increased secretion of cortisol from the HPA axis during a stressful situation is essential for adaptation to the stressful situation. In addition, cortisol mediates inactivation of the HPA axis by negative feedback. Cortisol exerts its effect via binding to a receptor. There are two receptor types for cortisol; the mineralocorticoid receptor (MR), having a high affinity and the glucocorticoid receptor (GR), having a low affinity for the naturally occurring glucocorticoid. In the brain the MR is continuously occupied with cortisol while the GR only becomes activated at high concentrations of cortisol. Both the MR and GR play an important role in the regulation of the HPA axis and are important for behavioral adaptation.

The ultimate goal of the research described in this thesis was to investigate whether genetic variation in the mineralocorticoid receptor is involved in the development of depression.

First, the coding region of the MR gene was screened for genetic variation and using computer programs linkage between the genetic variation and the possible functionality of the genetic variation was tested; this is described in **chapter 2**. There were seven Single Nucleotide Polymorphisms (SNPs) located in the coding region of the MR-gene. A SNP is a variation in one nucleotide with a frequency of more than 1% in the population. The SNPs in the MR gene are mostly linked and several SNPs are putatively functional. The putative functional SNPs are MR-2G/C (rs20170951), MRI180V (rs5522) and rs2871. MR-2G/C is located just outside the coding region of the gene in the Kozak region and the SNP might therefore influence the translation of the mRNA to protein. MRI180V is located in the coding region of the gene in exon 2 and changes the amino acid on position 180 from an isoleucine to a valine. Rs2871, one of the SNPs in the non-coding domain of exon 9, is located in a part of the mRNA that is prone to loop formation and this might have consequences for the stability of the mRNA.

Based on the location and putative function, the SNPs MR-2G/C and MRI180V were selected for further research. Functionality of these SNPs was tested with *in vitro* cell systems. The results of the *in vitro* tests are described in different chapters of this thesis. The C allele of MR-2G/C results in

more MR protein compared to the G allele, while the amount of MR mRNA was not influenced, this was described in **chapter 4**. The increased MR expression with the C allele of MR-2G/C lead to higher transactivation as descibed in **chapter 3 and 4** and increased ligand binding, described in **chapter 5**. MRI180V had previously been tested in transactivation assays, the V allele decreased transactivation without changing the expression or ligand binding (**chapter 5**).

MR-2G/C and MRI180V were, to a large extent, linked to each other, as described in **chapter 2, 3, 5 and 6**. The combination of two (partly) linked SNPs is called a haplotype. Without linkage 4 different combinations (haplotypes) are possible. In our studies, however, only 3 combinations (haplotypes) were observed. The combination MR-2G and MR180V (=G nucleotide) did not occur. To exclude the possibility that the two SNPs influence each other's functionality, the haplotypes were also tested *in vitro*, this was described in **chapter 5**. The two SNPs did not influence each other *in vitro*.

In each chapter an association study was performed between genetic variation and one of the possible consequences of the variation.

In **chapter 3** involvement of the two *in vitro* functional SNPs MR-2G/C and MRI180V on the regulation of the HPA axis in healthy individuals was tested by using the dexamethasone suppression test (DST). Dexamethasone is a synthetic glucocorticoid and, like high concentrations of cortisol, it is able to suppress the HPA axis. In our study a low dose dexamethasone (0.25mg) was used and this resulted in decreased but not completely suppressed cortisol levels the day after intake. The peak in cortisol lasting one hour just after awakening was not influenced by the SNPs without dexamethasone. However, after the intake of dexamethasone the previous evening there was a clear effect of the SNPs on cortisol levels and this effect was gender specific. The decrease was highest in women with the MR-2G/C GG genotype, while men with this genotype showed an increase compared to the men with other genotypes. Men with the MRI180V AA genotype had higher cortisol concentrations than men with the AG genotype, while this SNP had no effect in women. Haplotype analysis did not reveal additional information; the effect was mainly mediated by MR-2G/C.

In **chapter 4** the influence of MR-2G/C on blood pressure and salt regulation was tested. In addition to the important role of the MR in the HPA axis, the MR is also important in de regulation of salt and blood pressure via the renine angiotensine system (RAS). The hormone aldosterone binds the MR receptor in the kidney and this results in water and salt retention The G allele of MR-2G/C was associated with increased activation of the RAS and higher blood pressure.

In **chapter 5** the influence of the MR haplotypes containing MR-2G/C and MRI180V on the response to an acute psychosocial stressor and on chronic stress was tested. Individuals homozygous for the haplotype containg the MR-C and MRI180 alleles had the highest saliva cortisol, plasma ACTH and heart rate during the psychosocial stressor. This haplotype resulted in vitro in the highest MR expression and transactivation. Chronic stress was measured with questionaires and the subscales "social isolation" and "work overload" were associated with the MR haplotypes, however this was mediated by the haplotype with the MR-C and MR180V alleles.

Individuals carrying this hapotype reported more chronic stress. The set-up of this study was hypothesis generating; the results suggest that more MR protein leads to higher responses on acute psychosocial stress and that MR haplotypes influence the perception of chronic stress.

In **chapter 6** two MR haplotypes were tested and described in a cohort consisting of one hundred patients with mood and/or anxiety disorders and fifty healthy individuals. Due to the relatively low number of patients and controls this study was also explorative in nature and hypothesis generating. Association studies were performed with haplotypes in the beginning of the gene (5', -2 G/C en 180) and with haplotypes at the end of the gene (3', in exon 9). The two haplotypes were not associated with mood and/or anxiety disorders but in the patient group there was an association with the personality trait neuroticism.

Chapter 7 is the general discussion of the thesis.
First, the location of the SNPs was analyzed. MR SNPs were not located throughout the gene, they were mainly located in the promoter region, exon 2 and at the end of exon 9. Genetic changes on other locations are probably too severe to be spread through the population.
Secondly, the predictive value of the *in vitro* studies was discussed. The *in vitro* functional SNPs were associated with different measures *in vivo,* therefore it seems important to perform in vitro studies. However, generating a detailed hypothesis for *in vivo* associations based on the *in vitro* results appeared to be complicated. This is illustrated by the gender differences *in vivo*. Many associations were gender specific, the effects being different in men and women. Some associations were in the completely opposite direction between men and women. In the current *in vitro* assays there are no sex hormones present. Adding sex hormones in the *in vitro* studies or testing different age groups *in vivo* might clarify the associations. These and other recommendations for future studies are described in **chapter 7**.

In this thesis the genetic variation in the MR was described. There is genetic variation in the MR and this variation is functional in *in vitro* studies. The genetic variation was associated with cortisol levels after dexamethasone, the reaction on psychosocial stress, chronic stress and neuroticism. Due to the relatively low number of individuals in each study, these studies were explorative and the associations found need follow up testing. We postulate that genetic variation in the MR modulates the stress response and affects in part a person's personality. Furthermore, the genetic variant MR-2G/C was associated with blood pressure and salt regulation and we were able to confirm this in different cohorts.

Samenvatting

Een depressie is een stemmingsstoornis die gekenmerkt wordt door verlies aan levenslust en een zwaarmoedige stemming. Veel mensen hebben wel eens een dipje maar pas als deze gevoelens langdurig aanhouden zou er sprake kunnen zijn van een depressie. Een depressie is een veel voorkomende ziekte en de ziekte belemmerd normaal functioneren. In Nederland krijgt 15% van de bevolking gedurende zijn leven een of meerdere depressieve episodes. Tijdens deze periodes is het vaak niet mogelijk te werken of studeren. Factoren die betrokken zijn bij het ontstaan van de ziekte en het mechanisme achter de ziekte zijn veelvuldig onderzocht maar nog steeds niet volledig achterhaald. Het is wel bekend dat stress een belangrijke rol speelt. Ook zijn sommige families vatbaarder en dat duidt op een genetische component. Tweelingen studies waarbij 100% genetisch identieke een-eiige tweelingen vergeleken werden met twee-eiige tweelingen die genetisch gemiddeld 50% identiek zijn bevestigen inderdaad dat er een genetisch component aanwezig is bij het ontstaan van een depressie.

Van groot belang is de vondst dat een depressie geassocieerd is met een verstoring van de Hypothalamus-Hypofyse-Bijnier-as (HHB-as). Dit is een belangrijk systeem in het lichaam dat de reactie op stress coördineert en reguleert. Tijdens stress is de verhoogde secretie van cortisol door verhoogde activiteit van de HHB-as van essentieel belang voor de aanpassing aan de stressvolle situatie. Cortisol zorgt er via negatieve terugkoppeling ook voor dat het systeem zelf weer uitgeschakeld wordt. Cortisol oefent zijn effect uit via binding aan een cortisol receptor. Er bestaan twee typen receptoren voor cortisol; de hoog affine zogenoemde mineralocorticoïd receptor (MR) en de laag affine glucocorticoïd receptor (GR). De MR in de hersenen is vrijwel altijd bezet door cortisol, terwijl de GR pas geactiveerd wordt als er hoge concentraties cortisol in het lichaam circuleren. Deze twee receptoren voor cortisol, de GR en MR spelen een centrale rol in de regulatie van de HBB-as en zijn daarnaast van groot belang voor gedragsmatige adaptatie.

Het doel van het onderzoek beschreven in dit proefschrift was te onderzoeken of genetische variatie in de mineralocorticoïd receptor betrokken is bij het ontstaan van een depressie.

Eerst werd het coderende gedeelte van het MR gen gescreend op de aanwezigheid van genetische variatie. Vervolgens werd met computerprogramma's de koppeling tussen de genetische variatie en de mogelijke functionaliteit van de genetische variatie getest. Dit is beschreven in **hoofdstuk 2**. Er bleken zeven Single Nucleotide Polymorphisms (SNPs) aanwezig te zijn in het coderende gedeelte van het MR gen. Een SNP is een variatie in het DNA die veelvuldig voorkomt in de populatie en waarbij 1 nucleotide veranderd is. De genetische variatie in het MR gen bleek voor een groot deel aan elkaar gekoppeld te zijn en bovendien bleken een aantal SNPs mogelijk functioneel. Enkele mogelijk belangrijke SNPs zijn MR-2G/C (rs20170951), MRI180V (rs5522) en rs2871. De SNP MR-2G/C bevindt zich net buiten het coderende gedeelte in de Kozak regio (belangrijk bij eiwit productie) van het gen en beïnvloedt mogelijk de translatie van het mRNA naar eiwit. De SNP MRI180V bevindt zich in het coderende gedeelte van het gen in exon 2 en verandert het aminozuur

op positie 180 van een isoleucine naar een valine. Tenslotte, rs2871, één van de SNPs in het niet coderende gedeelte van exon 9, bevind zich in een stuk mRNA dat een 'loop' kan vormen en dit heeft mogelijk gevolgen voor de stabiliteit van het mRNA.

De SNPs MR-2G/C en MRI180V werden op basis van de locatie en mogelijke functie geselecteerd voor verder onderzoek. De functionaliteit van deze twee SNPs werd getest met *in vitro* cel systemen. De resultaten van de *in vitro* testen zijn in verschillende hoofdstukken van dit proefschrift beschreven. Het C allel van MR-2G/C geeft in de *in vitro* cel systemen meer eiwit dan het G allel zonder dat de hoeveelheid mRNA beïnvloed werd, zoals beschreven in **hoofdstuk 4.** De verhoogde MR expressie met het C allel leidde vervolgens tot een verhoogde transactivatie, beschreven in **hoofdstuk 3 en 4** en een verhoogde ligand binding, beschreven in **hoofdstuk 5.** De invloed van MRI180V op transactivatie was reeds getest. Het V allel verminderd de transactivatie dit blijkt te gebeuren zonder dat de MR expressie of de ligand binding wordt beïnvloed (**hoofdstuk 5).**

MR-2G/C en MRI180V bleken voor een deel aan elkaar gekoppeld, dit werd beschreven in **hoofdstuk 2, 3, 5 en 6**. De combinatie van twee (gedeeltelijk) gekoppelde SNPs wordt een haplotype genoemd. Zonder koppeling zijn er 4 verschillende combinaties (haplotypes) mogelijk terwijl in onze studies maar 3 combinaties (haplotypes) geobserveerd werden, de combinatie MR-2 G en MRI180 V (=G nucleotide) kwam niet voor. Om uit te sluiten dat de SNPs elkaars functionaliteit beïnvloeden werden ook de haplotypes *in vitro* getest, dit is beschreven in **hoofdstuk 5.** De twee SNPs bleken elkaar niet te beïnvloeden in de gebruikte tests.

Per hoofdstuk werd er met associatie-studies gekeken naar één van de mogelijke functies van genetische variatie in de MR.

In **hoofdstuk 3** werd gekeken of de *in vitro* functionele SNPs MR-2G/C en MRI180V de regulatie van de HHB-as kunnen beïnvloeden in gezonde vrijwilligers. Hiervoor werd gebruik gemaakt van de dexamethason suppressie test (DST). Dexamethason is een synthetisch gucocorticoïd en kan, net als hoge hoeveelheden cortisol, de cortisol productie door de HBB-as onderdrukken. Met dexamethason wordt de negatieve terugkoppeling van de HHB-as getest. In onze studie werd gebruik gemaakt van een lage dosering dexamethason (0.25mg) en dit leidde tot verminderde maar niet geheel onderdrukte cortisol spiegels de dag na inname. De één uur durende piek in cortisol na wakker worden werd zonder dexamethason niet beïnvloed door de SNPs maar na inname van dexamethason was er wel een een duidelijk effect van beide SNPs aanwezig. Dit effect was afhankelijk van het geslacht van de vrijwilliger. De onderdrukking was het sterkst in vrouwen met het MR-2G/C GG genotype terwijl mannen met dit genotype juist een verhoging lieten ziet t.o.v. mannen met de andere genotypes. Mannen met het MRI180V AA genotype hadden hogere waardes dan mannen met het AG genotype terwijl deze SNP geen effect had in vrouwen. Haplotype analyse leverde geen aanvullende informatie op, het effect werd voornamelijk veroorzaakt door MR-2G/C.

In **hoofdstuk 4** werd de invloed van MR-2G/C op zout regulatie en bloeddruk getest. Behalve de belangrijke rol van de MR in de HHB-as heeft de MR ook een belangrijke rol in zout en bloeddrukregulatie via het renine angiotensine systeem (RAS). Het hormoon aldosteron bindt aan

de MR in de nier en leidt tot water- en zout retentie. Het G allel was geassocieerd met toegenomen activatie van het RAS en hogere bloeddruk.

In **hoofdstuk 5** werd onderzocht of de MR haplotypes, die MR-2G/C en MRI180V bevatten, de reactie op acute psychosociale stress kunnen beïnvloeden en of deze haplotypes geassocieerd waren met chronische stress. Individuen homozygoot voor het haplotype dat de MR-C en MRI180 allelen bevat hadden de hoogste speeksel cortisol, plasma ACTH en hartslag reactie tijdens de psychosociale stressor. Dit haplotype gaf *in vitro* de hoogste expressie van het MR eiwit en de hoogste transactivatie. Chronische stress werd gemeten met vragenlijsten en de subschalen "sociale isolatie" en "hoge werkdruk" bleken geassocieerd met de MR haplotypes, dit keer was het het echter het haplotype met de allelen MR-C en MR180V die in dragers van dit haplotype tot meer chronische stress leidde. Deze studie was hypothese genererend van opzet en suggereert dat meer MR eiwit tot hogere reacties op acute psychosociale stress leidt en dat MR haplotypes betrokken zijn bij de verwerking van chronische stress.

In **hoofdstuk 6** werden twee MR haplotypes beschreven en getest in een kleine groep van honderd patiënten met angst en/of stemmingstoornissen en vijftig gezonde vrijwilligers. Vanwege de relatief kleine aantallen patiënten en controles was ook deze studie exploratief en hypothese genererend van opzet. Associatie-studies werden uitgevoerd met haplotypes in het begin van het gen (5', -2 G/C en 180) en met haplotypes in het einde van het gen (3', in exon 9). De twee haplotypes waren niet geassocieerd met angst en/of stemmingstoornissen maar in de patiënten groep was wel een associatie met het persoonlijkheidskenmerk neuroticisme.

Hoofdstuk 7 is de algemene discussie van het proefschrift.

Eerst werd de locatie van de MR SNPs geanalyseerd, MR SNPs bleken niet in het gehele gen aanwezig te zijn, maar voornamelijk voor te komen in het promoter gebied, exon 2 en aan het eind in exon 9. Genetische veranderingen op andere locaties veroorzaken waarschijnlijk ernstige aandoeningen.

Vervolgens werd de voorspellende waarde van *in vitro* functionaliteits studies voor effecten *in vivo* bediscussieerd. De *in vitro* functionele SNPs bleken zoals verwacht geassocieerd te zijn met *in vivo* effecten; het daarom zinvol om *in vitro* studies uit te voeren. Echter een gedetailleerde hypothese genereren op basis van *in vitro* gegevens blijft moeilijk, wat geïllustreerd wordt met de man – vrouw verschillen *in vivo*. Bij veel associaties waren sexe verschillen in de effecten aanwezig en in sommige gevallen was de associatie zelfs tegenovergesteld bij mannen vergeleken met vrouwen. In de huidige *in vitro* testen zijn geen geslachtshormonen toegevoegd om de verschillen tussen mannen en vrouwen te onderzoeken en het is dus niet mogelijk om deze effecten te voorspellen. In vervolgstudies zou het effect van een geslachtshormoon in de *in vitro* studies onderzocht dienen te worden. Ook zou onderzoek aan andere leeftijdsgroepen in de associatie- studies een optie kunnen zijn. Deze suggestie maar ook andere suggesties voor vervolgonderzoek zijn in **hoofdstuk 7** beschreven.

Kort samengevat, in dit proefschrift werd genetische variatie in de MR beschreven. Er is genetische variatie in de MR aanwezig en deze variatie is functioneel in *in vitro* studies. De genetische variatie was geassocieerd met cortisolspiegels na dexamethason, de reactie op psychosociale stress, gedurende chronische stress en bij neurotisisme. Gezien de kleine groepen deelnemers in de studies waren deze studies exploratief en zouden de gevonden associaties verder bestudeerd moeten worden. Wij postuleren dat genetische variatie betrokken is bij de reactie op stress en in gedrag. Verder bleek de genetische variant MR-2G/C geassocieerd te zijn met bloeddruk en zoutregulatie; deze laatste associatie hebben we reeds kunnen bevestigen in meerdere cohorten.

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Publications and Presentations

Publications

Functional Mineralocorticoid Receptor (MR) gene variation influences the cortisol awakening response after dexamethasone ***N. van Leeuwen****, R. Kumsta, S. Entringer, E.R. de Kloet, F.G. Zitman, R.H. DeRijk, & S. Wüst* Psychoneuroendocrinology 2010, 339-349

The functional -2 C variant of the Mineralocorticoid receptor modulates basal and salt-dependent rennin and aldosterone levels and associates with lower blood pressure ***N van Leeuwen*,*** *M Caprio*, C Blaya, F Fumeron, P Sartorato, G Giacchetti, F Mantero, FL Fernandes-Rosa, C Simian, S Peyrard, FG Zitman, R de Kloet, M Azizi, X Jeunemaitre, RH DeRijk & MC Zennaro* Hypertension, epub 20 September 2010

Corticosteroid receptor- gene variants: Modulators of the stress-response and implications for mental health. *R.H. deRijk,* ***N. van Leeuwen****, M.D. Klok, F.G. Zitman* European Journal of pharmacology 2008, 492-50

Human Mineralocorticoid Receptor (MR) gene haplotypes modulate MR expression and transactivation: implication for the stress response. ***N. van Leeuwen*****, S. Bellingrath*, E.R. de Kloet, F.G. Zitman, R.H. DeRijk, B.M. Kudielka & S. Wüst* Psychoneuroendocrinology (*in press*)

Mineralocorticoid Receptor gene-variants as determinants of HPA axis regulation and behavior. *R.H. DeRijk, E.R.de Kloet, F. G. Zitman and* ***N. van Leeuwen*** Endocrine book series, 2010 (*in press*)

Presentations

MR gene variants *in vitro* and *in vivo.* Marius Tausk Masterclass 2009 Oegstgeest

An *in vitro* functional mineralocorticoid receptor (MR) gene variant is associated with the cortisol response to psychosocial stress. Spring School the ABC of Stress 2009 Dresden Germany

An *In vitro* functional SNP in the human Mineralocorticoid Receptor (MR) is associated with the cortisol awakening response (CAR) after 0.25mg dexamethasone. ISPNE 2008 Dresden, Germany

Mineralocorticoid receptor (MR) gene variants modulate the CAR. Dutch EndoNeuroPsycho meeting meeting 2008 Doorwerth

Stress: de invloed van genetische variatie in de mineralocorticoid receptor (MR). NVVP 2008 Amsterdam

Functionele polymorfismen in de mineralocorticoid receptor in stress respons en psychopathologie. NVVP 2007 Maastricht

The role of single nucleotide polymorphisms (SNPs) in the Mineralocorticoid Receptor (MR) in stress response and psychopathology. First prize poster competition Dutch EndoNeuroPsycho meeting meeting 2007 Doorwerth

Functional analysis of human mineralocorticoid receptor variants associated with stress responsiveness. Dutch EndoNeuroPsycho meeting meeting 2006 Doorwerth

Curriculum vitae

Nienke van Leeuwen werd op 22 december 1975 geboren in Haarlem. In 1993 behaalde zij haar HAVO diploma aan het Linnaeus college te Haarlem. In 1993 begon zij aan de HBO opleiding medische biologie aan de Hogeschool van Amsterdam waar zij in 1997 het diploma behaalde. Haar afstudeer stage heeft zij uitgevoerd bij TNO, afdeling preventie en gezondheid onder leiding van Dr. I. Meulenbelt en Prof. dr. P.E. Slagboom. Vervolgens is zij in 1997 met de studie biologie begonnen aan de Vrije Universiteit in Amsterdam waar zij in 1998 haar bachelor en in 1999 haar doctoraal diploma behaalde in de afstudeer richting medische biologie. Haar afstudeer stage van deze studie heeft zij uitgevoerd op de Vrije Universiteit, afdeling oncologie onder leiding van Dr. V.W. van Beusechem en Prof. dr. W.R. Gerritsen. Voor zij in 2005 begon met het in dit proefschrift beschreven promotie onderzoek bij de afdeling Medische Farmacologie van het Leiden/ Amsterdam Center for Drug Research (LACDR) en het Leids Universitair Medisch Centrum (LUMC) onder leiding van Prof. dr. E.R. de Kloet, Prof. Dr. F.G. Zitman en Dr. R.H. de Rijk is zij werkzaam geweest bij Crucell en psychiatrisch ziekenhuis Rivierduinen. Na haar promotie onderzoek is zij twee maanden blijven werken bij de afdeling Medische Farmacologie waar zij onder leiding van Dr. E. Vreugdenhil microRNA technieken opgezet heeft. Sinds december 2010 werkt zij bij de afdeling Moleculaire Celbiologie van het LUMC onder leiding van Dr. L.H. ’t Hart. Hier doet zij onderzoek naar de gentica van type 2 diabetes. Met behulp van *in vitro* technieken probeert zij het moleculaire mechanisme van recent geïdentificeerde diabetes genen te achterhalen. Bovendien voert zij in een groot cohort met diabetes patiënten farmacogenetisch onderzoek uit om na te gaan of het succes van de behandeling voorspeld kan worden op basis van het genotype van een patiënt.

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