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

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General Introduction

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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.

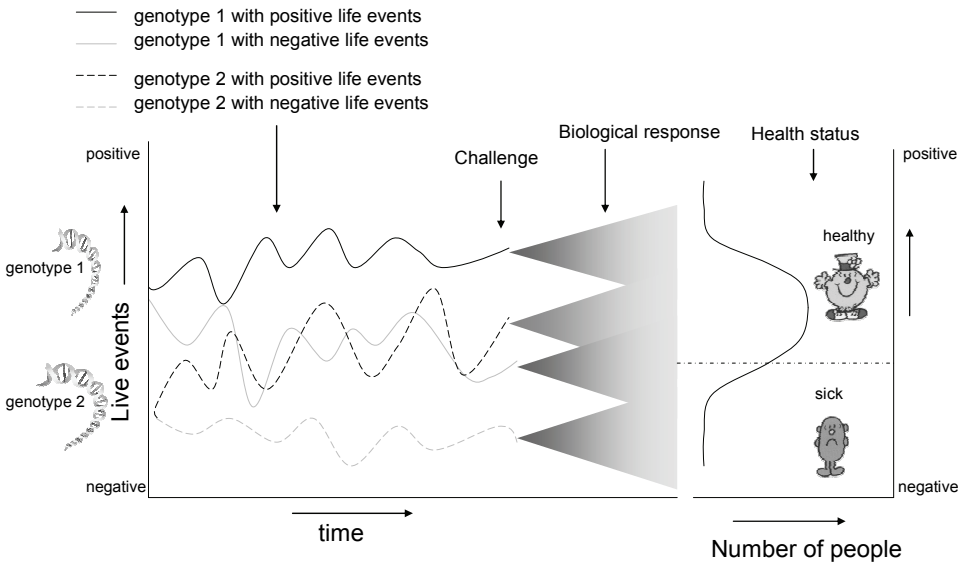


Figure 1. Schematic overview of the three hit model. Genetic variation (first hit) in interaction with life experiences (second hit) influences the response of brain and body following a challenge (third hit) later in life.

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.

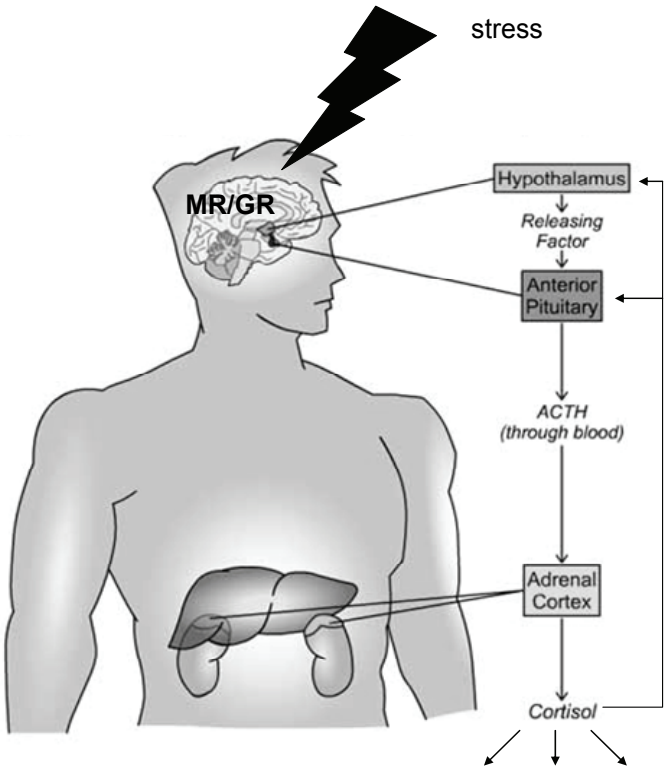


Figure 2. Schematic representation of the HPA-axis

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).

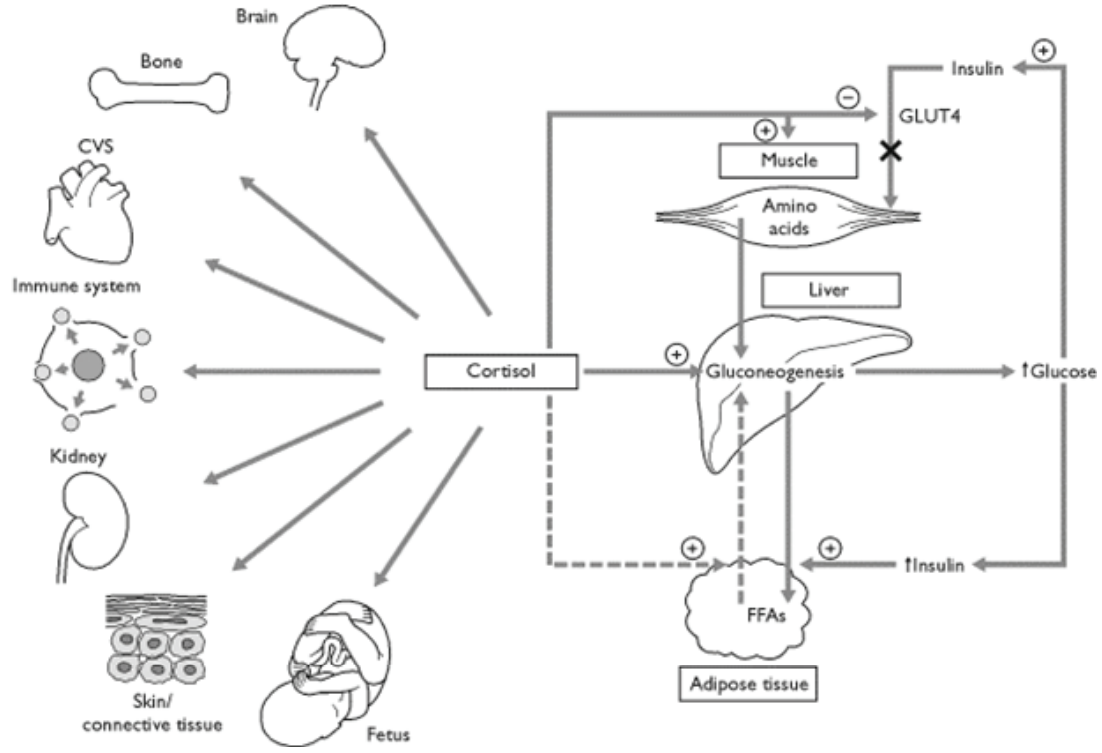


Figure 3. Schematic overview of cortisol target tissues.

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.

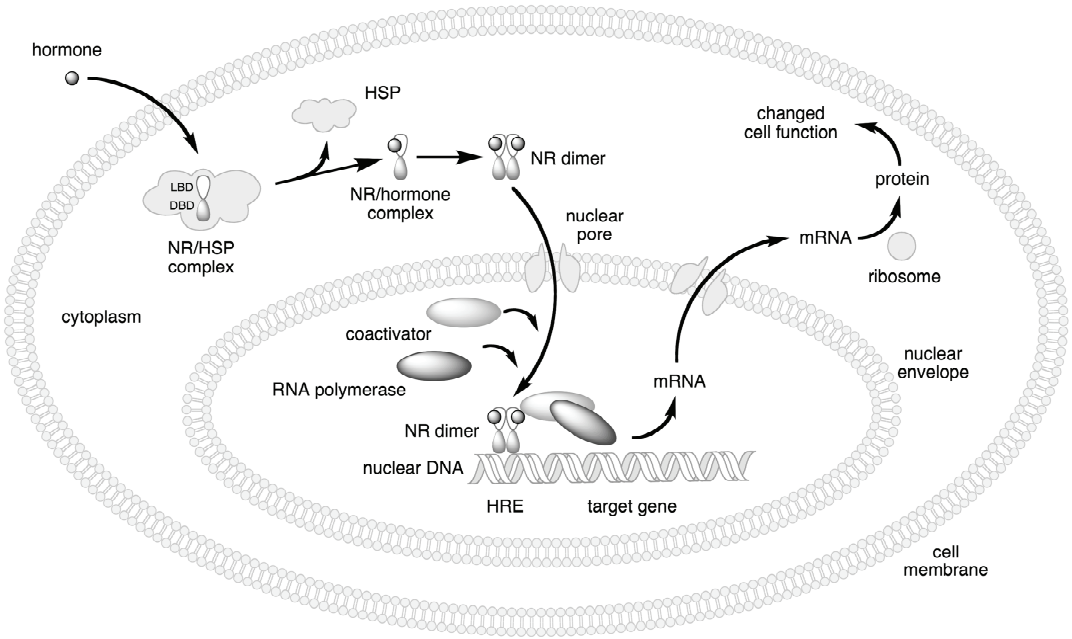


Figure 4. Cellular mechanism corticosteroid receptors. Ligand binding to a nuclear receptor (NR) e.g. GR or MR results in dissociation from the heat shock protein (HSP), dimerization, transloction from the cytoplasm to the nucleus, binding to specific sequences of the DNA called hormone response elements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

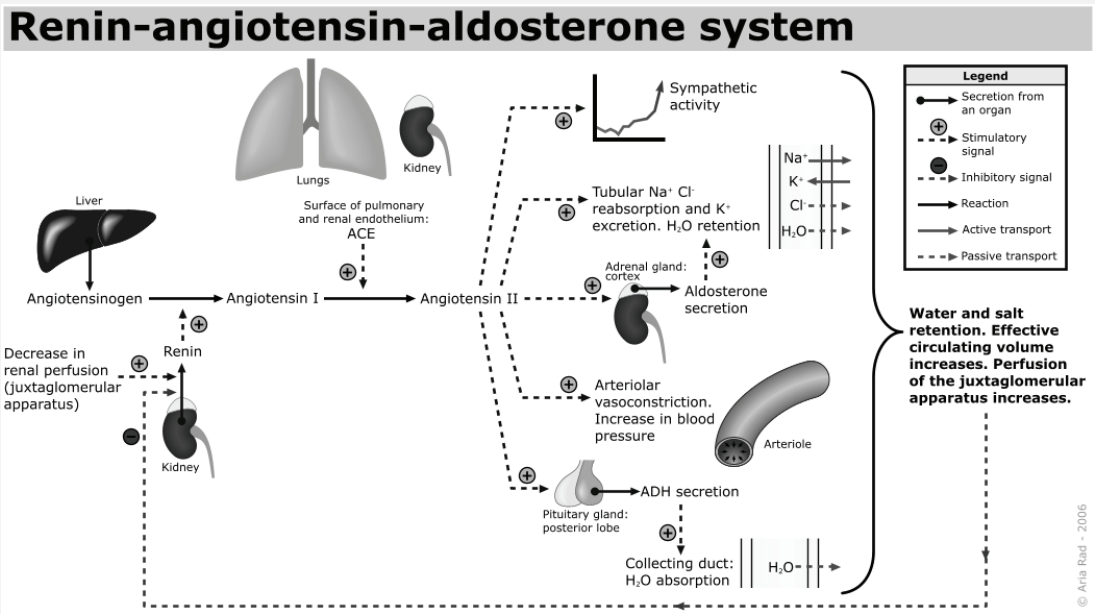
Box 1 The MR and the Renin Angiotensin System

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).

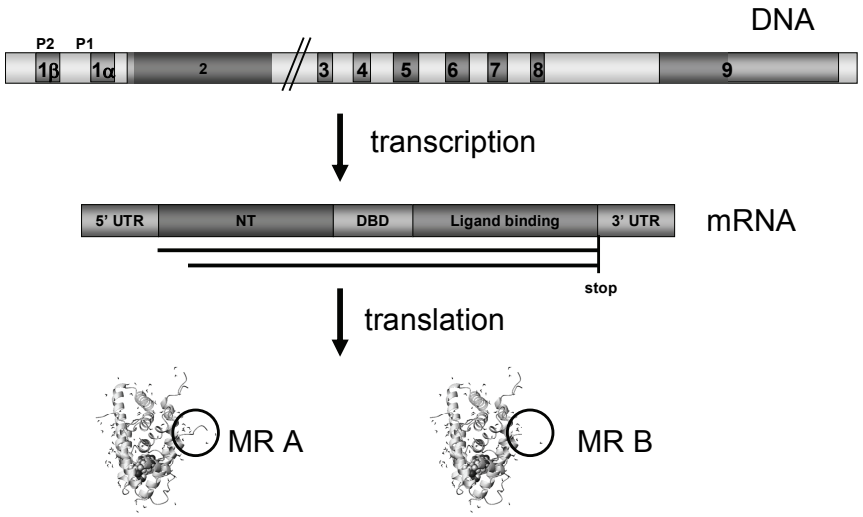


Figure 6.
Schematic representation of the human MR gene, MR mRNA and the two translational protein variants of the MR, named MR-A and MR-B. In the MR DNA the intronic regions are indicated in light gray, the dark gray areas indicate the exonic regions and correspond to the dark gray areas in the mRNA. 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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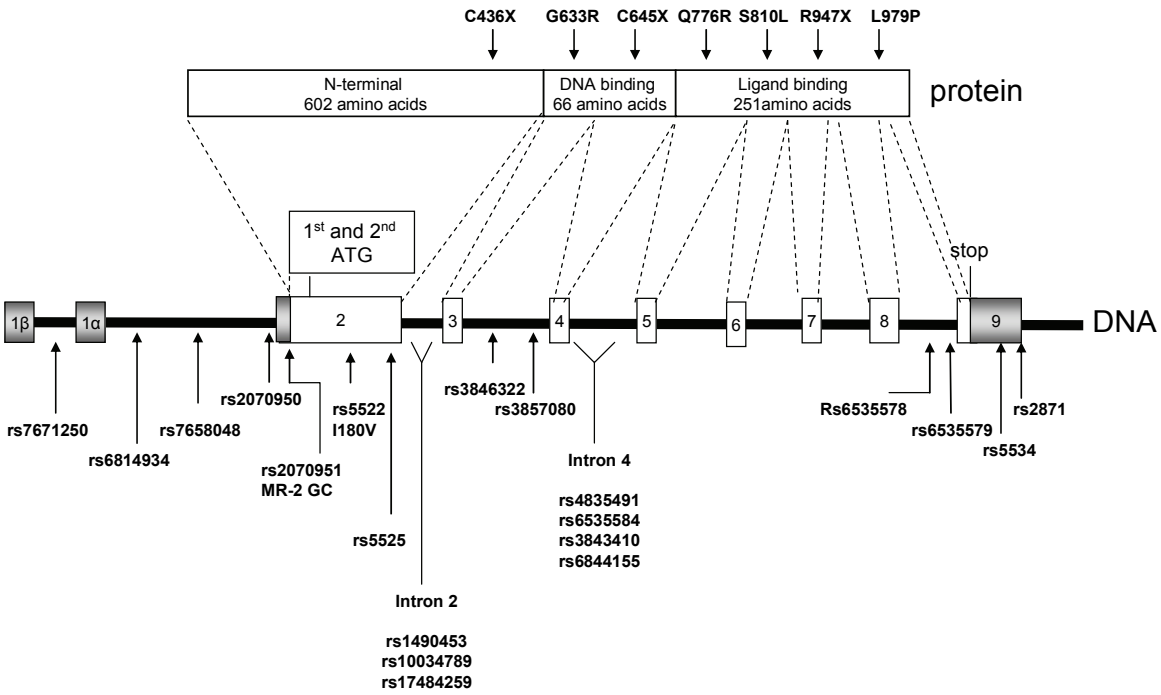


Figure 7. structure of the MR gene, intronic DNA regions are displayed as the black line in the middle, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA, while the protein structure and mutations are displayed above.

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.

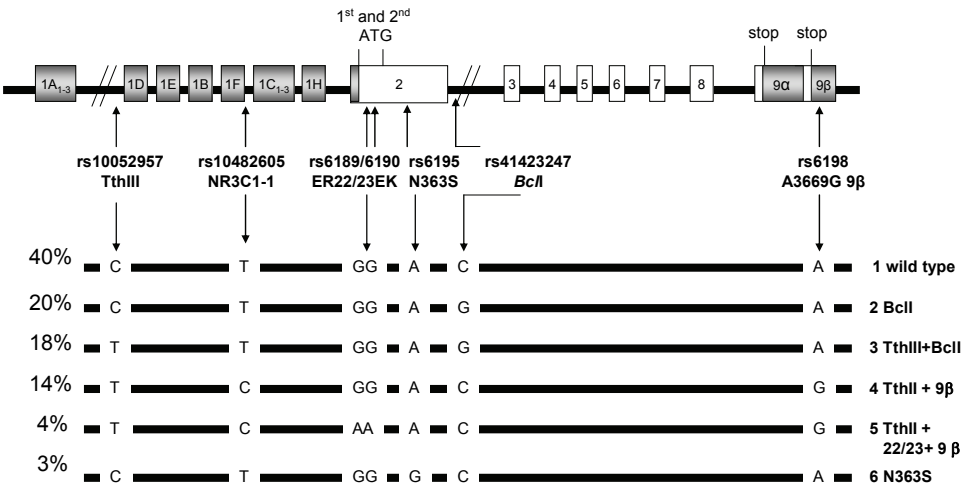


Figure 8. Structure and haplotypes of the hGR gene. Intronic DNA regions are displayed as the black line in the midle, exonic regions are displayed as the boxes on the line. SNPs and haplotypes are indicated below the DNA

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.

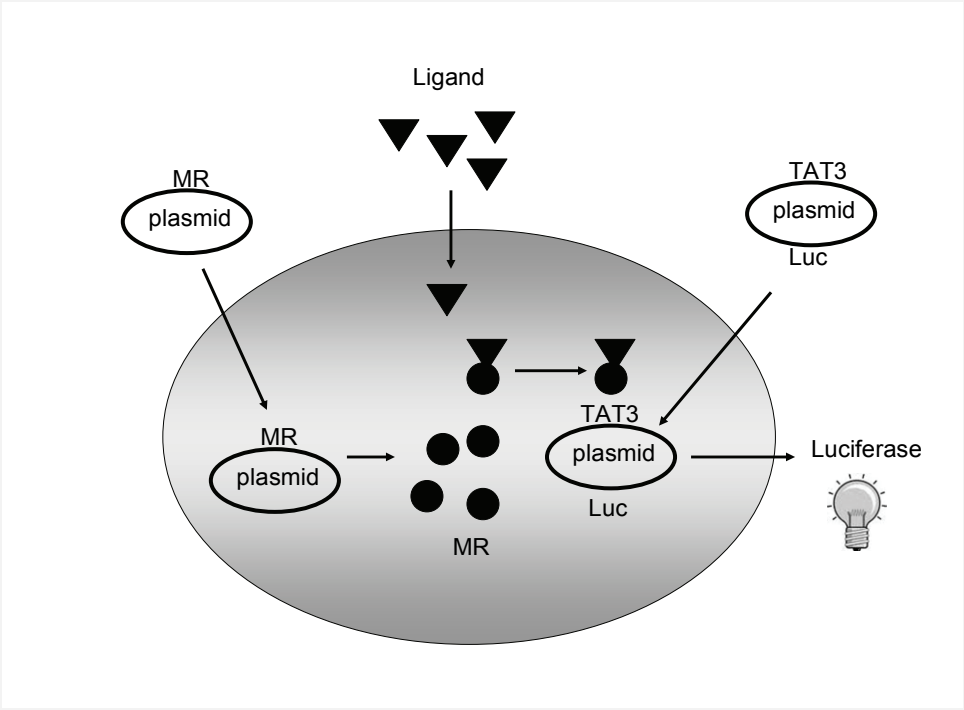
3. Scope and outline of this thesis

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**).