Mineralocorticoid receptor in human brain : a key player in resilience

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Chapter 1

General introduction

Outline

1.1 Stress response and the HPA axis

Every day an individual has to deal with challenges or stressors. A stressor, either of physical or psychological origin, elicits rapid changes in two interrelated communication systems in the body, the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos and Gold, 1992). With increased activity of the sympathetic nervous system adrenaline concentrations rise, causing increased metabolism, heart rate and blood pressure in order to provide tissues with oxygen and energy. Increased activity of the HPA axis (**Figure 1**) resulting in elevated blood levels of glucocorticoids (CORT, mostly cortisol in humans, corticosterone in rodents) coordinates with a somewhat slower onset more persistent effects among a broader spectrum of functions. In the short term, stress-induced cortisol/corticosterone secretion stimulates a wide variety of stress reactions, including immune function, pro-inflammatory reactions and cognitive and emotional processes, while inhibiting costly reproductive functions (de Kloet et al., 2005). The effects of cortisol on cognitive processes include appraisal and the actual choice of the most appropriate behavioral response to cope with a stressor (Oitzl and de Kloet, 1992; Brinks et al., 2007a), in part through its effects on neurotransmitters like excitatory and inhibitory amino acids (e.g. glutamate, GABA), serotonin and dopamine (Herman et al., 2003; Joels and Van Riel, 2004; Karst et al., 2005; Ambroggi et al., 2009; Di et al., 2009; Joels and Baram, 2009). It was only recently discovered that most of these rapid glucocorticoid effects are non-genomic (Joels et al., 2008).

Glucocorticoids influence emotional and cognitive processes by acting on numerous neuropeptide and neurotransmitter systems. Hence, genes that modulate HPA axis activity may indirectly modulate these systems.

After the rapid CORT actions, the glucocorticoid hormone dampens the very same processes that were initially promoted, while establishing negative feedback on the HPA axis itself in order to prevent them from overshooting and to become damaging (see also *HPA axis disturbance and its consequences*). This slower dampening action exerted by CORT includes the well-known immunosuppressive and anti-inflammatory effects, on which the wide therapeutic use of synthetic glucocorticoids like prednisone is based. In the brain this slower CORT action promotes learning and memory, preparing an individual for future encounters with similar stressful situations (Oitzl and de Kloet, 1992; de Kloet et al., 2005). Meanwhile, glucocorticoids promote metabolism by stimulating among others gluconeogenesis, providing glucose as an energy substrate to facilitate costly adaptation and recovery. Under resting conditions glucocorticoids facilitate the storage of energy supplies as readily available glycogen deposits. Hence the name glucocorticoids (Sapolsky et al., 2000a).

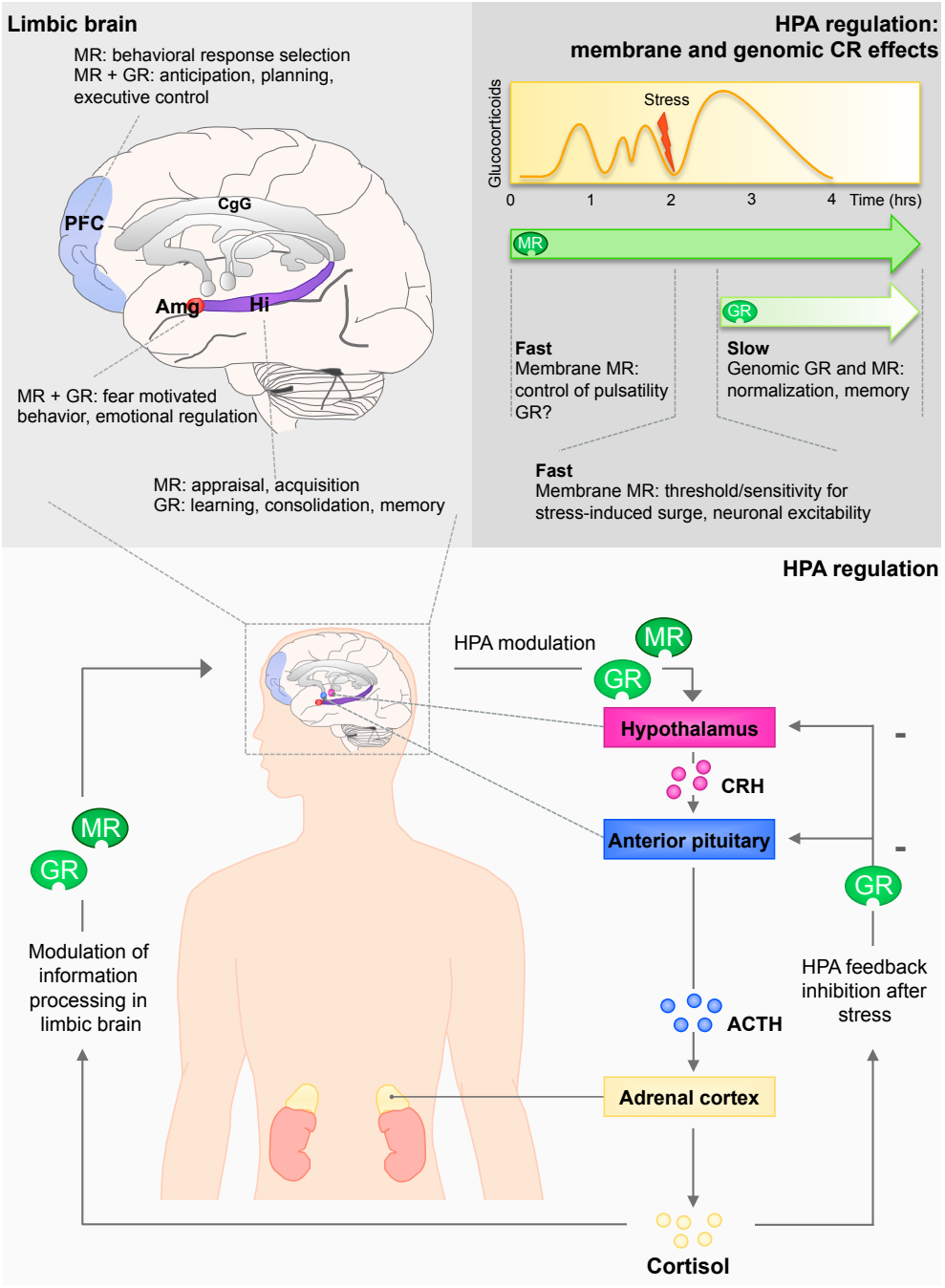


Figure 1 Schematic representation of HPA axis regulation, the limbic brain and the distinct functions of the MR and the GR

Box 1 HPA control during non-stress and stressful conditions

Non-stress (basal) HPA activity and the MR

HPA axis activity includes the sequential release of several potent hypothalamic, pituitary and adrenal hormones (**bottom Figure 1**). Parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) secrete corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophysial portal system. CRH will bind to CRH-1 receptors in the anterior pituitary gland, which will in turn release adrenocorticotrophic hormone (ACTH) into the bloodstream. Binding of ACTH to ACTH receptors (melanocortin-2 receptors, MC2R) in the adrenal cortex promotes the release of glucocorticoids (mainly cortisol in humans). Cortisol is not released constantly but in hourly pulses (Weitzman et al., 1971; Lightman et al., 2008; Lightman and Conway-Campbell, 2010). The amplitude of the pulses is the highest in the morning before the activity period starts and the lowest in the evening, resulting in a circadian rhythm over the ultradian cortisol pattern. During the night, the amplitude of the cortisol pulses slowly increases again. Upon awakening a profound pulse occurs, also known as the cortisol awakening response (CAR), which is superimposed on the circadian rhythm (Wilhelm et al., 2007). This cortisol surge lasts for around one hour, reaching peak levels at 30 minutes and returning to awakening levels at 60 minutes after awakening. The mineralocorticoid receptor (MR) has a high affinity for cortisol and is therefore always highly occupied throughout the day and plays an important role in the control of basal pulsatile HPA activity (**top right Figure 1**) (Reul and de Kloet, 1985; Young et al., 1998; Atkinson et al., 2008). It is known that at least in rodents also the glucocorticoid receptor (GR) is additionally activated at the ultradian cortisol pulses (Stavreva et al., 2009). The ultradian and circadian HPA axis/cortisol activity is important for coordination and synchronization of daily activities and sleep-related events. The recently discovered membrane variants of MR and GR have a lower affinity for cortisol.

Stress-induced HPA axis activity and the GR and MR

During stress the PVN receives inhibitory and excitatory inputs from limbic brain areas (**bottom Figure 1**; e.g. hippocampus, amygdala and prefrontal cortex) in order to process stressful cognitive and emotional information (Ulrich-Lai and Herman, 2009). Within a few minutes CRH, AVP and ACTH increase, with cortisol reaching maximum circulating levels at 15−30 minutes after the stressor. While corticosteroid levels increase also the GR becomes activated (Reul and de Kloet, 1985). It prevents the HPA axis from overshooting and mediates HPA negative feedback. Important to know is that also the MR plays a crucial role during stress. The MR and GR act in a complementary fashion to control distinct aspects of the HPA stress reaction (**top right Figure 1**). The MR maintains the threshold or sensitivity for stress-induced HPA activity, but is also implicated in the onset of the stress reaction and it modulates the rate of negative feedback (Oitzl et al., 1997; DeRijk et al., 2006). During the early phase of the stress reaction circulating levels of catecholamines, CRH/AVP and glucocorticoids rapidly increase. These signals further increase neuronal excitability, resulting in enhanced emotional arousal and MR-mediated (non-genomic) behavioral response selection. Then, during the later phases of the stress reaction the glucocorticoids start to have their slow genomic effects. Changes in expression of specific and partially overlapping glucocorticoid responsive target genes are mediated by the MR as well as the GR. At the cellular level the MR is responsible for maintenance of neuronal excitability and integrity, resulting in appraisal of the sensory information. The GR mediates normalization of cellular homeostasis and promotes the storage of the learned information (**top left Figure 1**). Recent research has demonstrated that the magnitude of the HPA, molecular and behavioral responses to stress varies over the ultradian cycle, with stronger responses occurring during the rising phase compared to the falling phase (Sarabdjitsingh et al., 2010a).

HPA axis regulation by cortisol is mediated by two types of receptors

The HPA axis involves the sequential release of hypothalamic and pituitary hormones, with adrenal cortisol being the end hormone (**Figure 1**; **Box 1**). Important players in feedback regulation of HPA axis activity by cortisol are two receptor types, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet, 1985). Both the MR and GR bind cortisol and mediate cortisol effects throughout the body and brain, including the negative feedback on stress-induced HPA axis activity. In this respect the MR and GR operate in a complementary fashion. Via the MR cortisol controls the initial reaction to a stressor by maintaining the sensitivity or threshold for stress-induced HPA activation, in a so-called 'proactive mode' and via the GR cortisol is involved in negative feedback in order to facilitate HPA deactivation and recovery from the stressor, the so-called 'reactive mode' (de Kloet and Reul, 1987; de Kloet et al., 1998). Control of HPA axis activity is critical for the maintenance of homeostasis and allostasis, if this cannot be established HPA axis activity remains high and this can have profound destructive effects on body and mind.

HPA axis disturbance and its consequences

With respect to emotional arousal and cognitive performance during stress, glucocorticoids seem to function according to an inverted U-shape, mediated by the high-affinity MR, the lower-affinity GR and the more recently discovered membrane bound lower-affinity MR and membrane bound GR (de Kloet et al., 1999; Johnson et al., 2005; Karst et al., 2005; Joels, 2006; Brinks et al., 2007b; Di et al., 2009; Karst et al., 2010). This means that too low or too high glucocorticoid levels may result in suboptimal functioning; a moderate cortisol response due to stress may have facilitating effects, while a very high increase in glucocorticoids can have disturbing effects on learning, memory and emotional processes (de Kloet et al., 1999; Brinks et al., 2007b). Still, this is largely context-dependent (Joels, 2006) (see also *Involvement of environment and genes in psychopathology*). Moreover, there is also the possibility of cortisol having damaging effects. This occurs for example when a persistent stressor is accompanied by chronic hypercortisolism. Under such conditions of chronic stress the costs to restore homeostasis may become too high, depleting energy resources, a condition called 'allostatic load' (McEwen and Wingfield, 2003). Chronic hypercortisolism can have damaging effects for example during the ageing process. Aged individuals may show a heightened and prolonged HPA response to a challenge (Kudielka et al., 2004), while some even suffer from hypercortisolism under non-stress conditions (Lupien et al., 1996). Hypercortisolism may promote neurodegenerative processes in the aged brain and has been correlated with hippocampal atrophy (Lupien et al., 1998). Together this may lead to cognitive disturbances, including problems with learning and memory and emotions (Lupien et al., 2005; Bremmer et al., 2007; Kuningas et al., 2007). HPA axis related disturbances among elderly were conceptualized into the 'glucocorticoid cascade hypothesis'; because of the inability to cope with a stressor an aged individual may end up in a vicious cycle of excess glucocorticoid release, neurodegeneration and disease (Sapolsky et al., 1986). Initially it was thought that only downregulation of the GR and consequent glucocorticoid resistance play a major role in this process. In the following sections it will become clear that this vicious cycle of hypercortisolism and disturbances in emotions and cognitive performance may as well be the result of aberrant MR functioning.

1.2 The HPA system in major depressive disorder

Many features of the classical 'glucocorticoid cascade hypothesis' (Sapolsky et al., 1986) that was based on the aging process apply to other stress-related processes, of which major depressive disorder has been most extensively studied.

HPA disturbances in major depressive disorder

Major depressive disorder (MDD) is a common disorder with a lifetime prevalence of around 15% in the Dutch population, striking women twice as often as men (Bijl et al., 1998). It is a complex disease with multiple neurobiological systems being disturbed, including the serotonin, norepinephrine and dopamine systems (Southwick et al., 2005). Also the HPA axis often shows disturbances, although results have been inconsistent (Stetler and Miller, 2005; Vreeburg et al., 2009a). There seems to be a nonlinear association between cortisol levels and symptoms of depression and anxiety (Wardenaar et al., 2011). Around 50% of the patients show HPA hyperactivity, with heightened levels of CRH in the CSF, ACTH in blood and cortisol in saliva, blood or urine. The HPA axis activity of depressed patients often escapes the dexamethasone (Dex)-suppression test (DST) or the combined Dex/CRH test. These tests assess the integrity of glucocorticoid negative feedback and the sensitivity for CRH at the level of the pituitary (reviewed in (Holsboer, 2000)). Dexamethasone, a synthetic glucocorticoid, normally results in HPA negative feedback through the GR, predominantly at the level of the anterior pituitary (de Kloet et al., 1974). CRH administration results in elevation of ACTH and cortisol. In both tests depressed subjects often show enhanced ACTH and cortisol release compared to healthy subjects. Finally, a heightened HPA response to a psychosocial stressor has been observed in MDD patients with a history of early-life adversity and chronic stress (Rao et al., 2008).

Several lines of evidence suggest that HPA disturbances resulting from glucocorticoid resistance and CRH hyperdrive underlie in part the pathophysiology and course of depression (Holsboer, 2000). Chronic stress has been associated with HPA disturbances as well as with the onset of depression (see also *Environmental factors*). It was postulated that the damaging effects of hypercortisolemia underlie the hippocampal volume loss and cognitive disturbances often observed in MDD patients (Sapolsky, 2000b), however, this is still debated (Fink, 2011). Several studies suggest that clinical relief after long-term treatment with antidepressants correlates with normalization of the HPA axis (Barden et al., 1995; Nickel et al., 2003; Zobel et al., 2004). In addition, an exaggerated cortisol response to the Dex/CRH test in remitted patients predicts relapse (Zobel et al., 2004). Furthermore, healthy family members of patients with depression show a heightened cortisol response to CRH (Holsboer, 2000). This indicates that HPA disturbance is a genetic trait that increases the risk of psychopathology.

HPA disturbances underlie in part the pathophysiology and course of depression.

MR/GR balance hypothesis of psychological dysfunction

As the MR and GR together are crucial for HPA control, emotions, cognitive processes and behavior, inappropriate activity of the GR as well as the MR may precipitate psychological disturbances. Several lines of animal research and studies among humans support this hypothesis. Altered central corticosteroid receptor expression has been reported for aged rodents as well as for adult and aged animals showing depressive-like behavior (Topic et al., 2008). Manipulation of MR or GR functioning by agonists and antagonists or by genetic modification in rodents may result in depressive- or anxiety-like behavior (Smythe et al., 1997; Gass et al., 2001). Rodents undergoing chronic stress during development show persistent changes in MR and GR expression, accompanied by changes in neuroendocrine and anxiety regulation (Sterlemann et al., 2008). Furthermore, distinct effects on behavior were found with differential MR or GR activation in rodents. While predominant MR activation, with or without low GR activation, was associated with low anxiety, high GR activation by chronic high corticosterone administration induces anxiety and cognitive problems (Brinks et al., 2007b). Another interesting finding was that long-term treatment of animals with antidepressants induces hippocampal MR and/or GR expression (Seckl and Fink, 1992; Lopez et al., 1998; Bjartmar et al., 2000). As already mentioned, some studies among patients suggested that clinical relief after long-term use of antidepressants correlates with HPA normalization (Barden et al., 1995; Nickel et al., 2003; Zobel et al., 2004). In addition, the clinical response to antidepressants among depressed patients is influenced by modulation of MR activity (Holsboer, 1999; Otte et al., 2010). Finally, several studies determined MR and/or GR expression in human *postmortem* brain tissue of depressed subjects, although most studies focused only on GR expression. Compared to healthy controls, MR or GR expression is different in multiple brain regions of the depressed subjects (Lopez et al., 1998; Webster et al., 2002; Knable et al., 2004; Xing et al., 2004; Wang et al., 2008; Alt et al., 2010). Together these data underscore the hypothesis of a MR/GR imbalance in depression (de Kloet et al., 1998; Holsboer, 2000). Three factors have been proposed through which MR/GR imbalance may arise; early-life experience, exposure to adult stressors and *MR* or *GR* gene variants (Oitzl et al., 2010). This is addressed in the following sections.

1.3 Involvement of environment and genes in psychopathology

Environmental factors

Numerous studies suggest a causal relationship between environmental (stressful) factors and psychopathology. Well-known examples are the experience of adverse life events, like death of a spouse or sexual or emotional abuse and daily hassles and the development of

depression (Kendler et al., 1999; de Graaf et al., 2002; Tennant, 2002; Hovens et al., 2010; Spinhoven et al., 2010). The consensus is that stressors have the most impact when there is lack of predictability and controllability, inducing the highest HPA responses and moderating behavioral coping style selection and health outcome (Kirschbaum and Hellhammer, 1994; Zakowski et al., 2001; Penley et al., 2002). Individuals that are particularly susceptible for stressful experiences during adulthood are subjects that have experienced trauma during their childhood. Early-life trauma may substantially affect the development of limbic brain areas and persistently disturb the HPA axis, making it difficult to appropriately cope with stress later in life (Heim et al., 2008; Lupien et al., 2009). However, also less severe environmental influences can have an impact on an individual's vulnerability, for example socioeconomic status (SES). Especially an individual's socioeconomic status during childhood can have consequences for health outcome in later life by having an impact on the development of an individual's personality and coping style (Heinonen et al., 2006).

Evidence from rodent studies shows that exposure to adult stressors and early-life adversity induce changes in MR and GR expression that are accompanied by profound effects on HPA functioning, behavior and emotional arousal (Sutanto et al., 1996; Gesing et al., 2001; Champagne et al., 2008; Sterlemann et al., 2008). Important to note here is that early-life adverse experiences may have maladaptive as well as adaptive effects in later life depending on later-life environment (Champagne et al., 2008), a finding that has led to the concept of 'match and mismatch' (Oitzl et al., 2010). Early-life experiences seem to program the brain and behavior in order to prepare an organism for similar encounters or situations later in life. However, HPA functioning and behavioral adaptations become compromised when there is a mismatch between early-life and later-life conditions. It is thought that the programming mechanism during early life involves epigenetic changes in which the GR might be an important target (McGowan et al., 2009).

Genetic factors

The chance to get depressed is higher when psychiatric disturbances are prevalent in the family. Indeed, twin studies indicate that the heritability of MDD is around 30-40% (Sullivan et al., 2000). A number of genes have been identified that are potentially implicated in the pathophysiology of depression (e.g. *FKBP5*, *BDNF*, *MAOA*, *COMT*, *5-HTT*), of which the serotonin transporter gene (*5-HTT*) has received most attention. Unfortunately, the genetic association studies performed thus far were inconclusive and even sometimes conflicting (Lasky-Su et al., 2005). Several reasons have been postulated to explain the failure to find consistent genetic associations with depression. First of all, patient groups are highly heterogeneous (see also *Use of endophenotypes*) and control populations are often selected in a different manner than the affected populations. With respect to SNPs, possibly more then a hundred independent SNPs may play a role and in different combinations, which could relate to the clinical heterogeneity. Moreover, some SNPs may have small effects, while others may have larger effects. Furthermore, other types of genetic variation occur, like copy number variants (CNVs), insertions, deletions and epigenetic variation (heritable differences in the genetic structure other than the DNA sequence, for example DNA methylation) (Maher, 2008). In addition, gene–gene interactions and gene–

environment interactions will greatly influence the final impact of a genetic variation on the phenotype.

Gene–environment interaction determines vulnerability versus resilience

The aforementioned research indicates that both environmental and genetic factors underlie psychopathology. However, a picture is emerging that it is the interplay between the environmental and genetic factors that precipitates a psychiatric disorder. A well-known example is the serotonin transporter gene (*5-HTT*) modulating the influence of stressful life experiences on the development of depression (Caspi et al., 2003). This study shows that a stressor can induce psychopathology depending on an individual's genetic makeup. In other words, a genetic vulnerability factor may increase the risk of psychopathology depending on the individual's experience of an adverse life event, a theory also described as the 'diathesis-stress' model. More recent studies take this model to a next level and propose a new model of 'differential susceptibility' (Belsky et al., 2009). This model encloses the more recent finding that 'risk alleles' are not just potential 'vulnerability genes' but can better be seen as 'plasticity genes'. Plasticity genes make an individual susceptible to the environment, for better or for worse. Under adverse circumstances a particular genetic variant may increase vulnerability for psychopathology, while in an enriched or supportive environment it may actually make an individual more resilient to psychopathology. Whether this model applies to all or just some of the genetic factors warrant further study. Nevertheless, the interplay between environment and genes modulates an individual's susceptibility, in the form of the resulting biological and/or psychological phenotype of the subject.

Genes don't act by themselves but need to be regulated. Environmental influences mediated by the HPA axis, its end product cortisol and its receptors, are extremely important for this purpose.

Use of endophenotypes

The complex etiology of psychiatric disorders hampers progress in the identification of genetic vulnerability or resilience factors. In part this is due to psychiatry's classification system, the fourth edition criteria of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV, American Psychiatric Association), which is at present still in use. This categorical classification system identifies MDD patients based on the presence of at least five listed clinical signs and symptoms. The result is that a group of patients diagnosed with MDD may include subgroups of patients with distinct clinical characteristics and biological causes. Moreover, psychiatric disorders are highly comorbid (de Graaf et al., 2002). Currently the DSM-V is under development, for which the inclusion of severity and comorbid dimensions is under consideration (http://www.dsm5.org). With respect to the biomedical research, an extensive number of studies are aimed to identify endophenotypes.

Endophenotypes include biochemical and endocrinological levels and psychological traits. The general idea is that endophenotypes are measurable markers that are genetically less complex than the clinical phenotype itself (Gottesman and Gould, 2003). Endophenotypes can be used to discriminate individuals at risk of psychopathology or to distinguish more homogenous subgroups of patients, which would increase the chance to identify common genetic vulnerability/resilience factors.

The present thesis will address several endophenotypes. One of them is the CAR (**Chapter 6**), which is thought to be in part genetically determined (Wust et al., 2000a). Other endophenotypes that will be addressed include the personality trait 'dispositional optimism' (**Chapter 4**) and cognitive reactivity to sad mood, with a special emphasis on thoughts of hopelessness (**Chapter 5**). While the first trait is thought to decrease the risk of depression, hopelessness is thought to increase the risk of depression and is often present in MDD patients. Heritability estimates of personality traits in general vary between 20−50% (Jang et al., 1996; Pilia et al., 2006).

Endophenotypes can be used to discriminate individuals at risk of psychopathology or to distinguish biologically more homogeneous subgroups of patients, with the goal to increase the probability to detect common genetic vulnerability factors.

1.4 Corticosteroid receptor gene variants

Functional single nucleotide polymorphisms in the glucocorticoid receptor (*GR*) gene
Because of its low affinity for cortisol the GR only becomes activated when cortisol levels are high, as occurs during stress and possibly during the ultradian pulses at the circadian peak (Stavreva et al., 2009; Sarabdjitsingh et al., 2010b). The effects of *GR* (*NR3C1*) gene variants are therefore preferably assessed in the context of stressful experiences. Multiple common single nucleotide polymorphisms (SNPs) and haplotypes have been identified (**Figure 2**) and tested in relation to a variety of functions. *In vitro* the NR3C1-1 SNP (rs10482605) located in the gene's promoter region affects gene transcription (Kumsta et al., 2009). The ER22/23EK polymorphism consists of two linked nucleotide changes in codons 22 and 23 (GAG AGG (GluArg) → GAA AAG (GluLys), rs6189 and rs6190) in exon 2 and *in vitro* this polymorphism results in a reduced capacity to activate target genes (Russcher et al., 2005). The N363S polymorphism located in codon 363 in exon 2 (AAT → AGT (AsnSer), rs6195) leads to an increased transactivation capacity (Russcher et al., 2005). Furthermore, the 9b SNP (rs6198) located in exon 9b increases stability of GRb mRNA and GRb protein expression (Derijk et al., 2001). Both the *TthIII*I SNP (rs100529570) in the promoter region and the *Bcl*I SNP (rs41423247) in intron B result in a restriction site change, but no *in vitro* data are available yet. SNPs that are functional *in vitro* potentially

affect gene functioning *in vivo*. Indeed, associations have been found between these *GR* gene variants and glucocorticoid sensitivity, HPA activity, metabolism, cardiovascular control and immune function (DeRijk et al., 2002; van den Akker et al., 2006; van Rossum and van den Akker, 2011). Moreover, associations were found between these SNPs (except for the N363S) and unipolar and/or bipolar depression (Spijker and van Rossum, 2009). With respect to cognition, thus far only the ER22/23EK polymorphism was found to associate with better attention performance in depressed patients (Spijker and van Rossum, 2009).

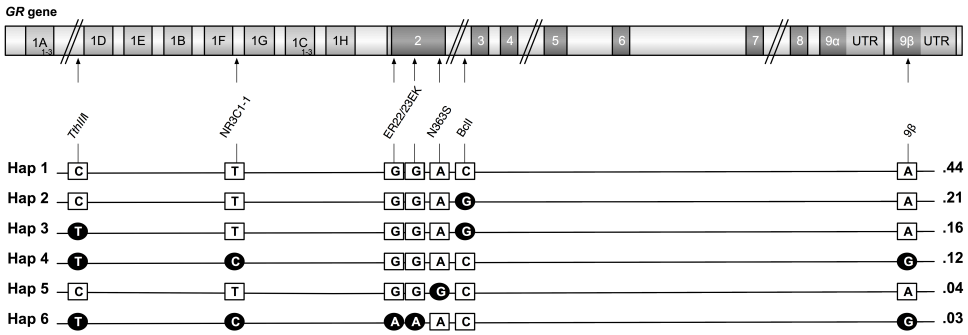


Figure 2 Schematic overview of the *GR* gene with its respective common and functional SNPs and haplotypes. Exons 1A to 1H and part of exon 9α or 9β lead to differential splicing and transcript expression (light grey boxes), while exons 2 to part of exon 9 are translated into protein (dark grey boxes). Nucleotide differences as compared to the most frequent haplotype are indicated (black ovals; haplotype structures and allele frequencies were adapted from (van Rossum et al., 2004; Derijk, 2009). Abbreviation: UTR, untranslated region.

Mineralocorticoid receptor expression and functions

In the periphery the MR is expressed mainly in epithelial cells of the kidney, colon and sweat glands and in non-epithelial cells of the heart and vascular wall (Zennaro et al., 1997). The MR is classically known for mediating the effect of aldosterone on the retention of Na+ in the kidney, hereby influencing volume control with consequences for blood pressure (Funder, 2005). In the kidney the MR binds aldosterone instead of cortisol due to the conversion of cortisol to receptor-inactive cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) (Edwards et al., 1988; Funder et al., 1988). In brain the 11βHSD2 enzyme seems to be mainly present in subregions of the brain stem and the subcommissural organ and is engaged in central regulation of the sodium balance (Seckl, 1997). Central expression of the 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) enzyme seems to be more widespread and acts as a reductase, converting the bio-inactive cortisone into cortisol. The MR therefore binds cortisol as well as aldosterone, but because the former steroid circulates in a much higher concentration, the MR predominantly *sees* cortisol. The MR has a 10 times higher affinity for cortisol than the GR (Reul and de Kloet, 1985). Therefore, the MR is always maintained highly occupied by the hourly pulses of glucocorticoids (Sarabdjitsingh et al., 2010b). The MR remains in the nucleus and is

potentially active under 'basal' or non-stressful conditions as well as under stressful conditions, suggesting that the receptor expression rather than the ligand is the rate-limiting factor. Recently however, also a membrane bound MR variant has been discovered which has a rather low affinity for glucocorticoids (see details below).

In agreement with animal studies it is known that in humans the highest MR expression can be found in the hippocampus, while also other limbic brain regions like the prefrontal cortex (PFC) and amygdala abundantly express MR (Pryce, 2008). Important to note is that MR expression is highly dynamic, showing changes for example during development (Vazquez et al., 1998), aging (van Eekelen et al., 1991; Topic et al., 2008), or after exposure to a physical or psychological stressor (Sutanto et al., 1996; Gesing et al., 2001; Macleod et al., 2003; Schmidt et al., 2004; Sterlemann et al., 2008; Topic et al., 2008). Several lines of evidence indicate that the MR modulates cortisol effects on emotions (see also *MR/GR balance hypothesis of psychological dysfunction*) and cognitive and behavioral flexibility, rendering the ability to select a different or even more appropriate strategy to deal with a novel situation (Oitzl et al., 1994; Berger et al., 2006; Otte et al., 2007; Schwabe et al., 2009). This makes the MR a highly interesting candidate gene to study in relation to psychological functioning.

Cortisol modulates via the MR emotions and cognitive and behavioural flexibility, making it a prime candidate gene to study in relation to psychological functioning.

Like the GR the MR was initially identified as an exclusive nuclear receptor. It resides in the cytoplasm, but after binding its ligand the MR translocates to the nucleus. There it acts as a transcription factor (TF) in order to influence gene transcription of glucocorticoid responsive genes (de Kloet et al., 1998; Datson et al., 2001). It seems that only a few of the glucocorticoid responsive genes are regulated by both the activated GR and MR, the majority is regulated by either the GR or the MR, indicating that the two receptors have to a large extend differential molecular effects (Datson et al., 2001). Importantly, effects of stress levels of cortisol on glutamate transmission and behavior can be established within minutes, while transcription takes about one hour. An 'alternative' MR was identified, localized in the neuronal membrane. This membrane MR was found to mediate fast non-genomic glucocorticoid effects on neuronal excitability. In hippocampal CA1 pyramidal neurons and basolateral amygdala the membrane MR enhances the frequency of miniature excitatory postsynaptic potentials (mEPSP), which is a measure for the probability of glutamate release (Karst et al., 2005; Joels et al., 2008; Karst et al., 2010). This finding suggests that through the MR and GR cortisol can coordinate fast non-genomic with the slower genomic effects and opens up a new field of research in HPA activity, behavior and emotions.

MR gene structure and splice variants

The gene encoding for the *MR* (*NR3C2*) is located on chromosome 4 (4q.31). The gene consists of 10 known exons (**Figure 3**). Translation of the gene into protein starts at exon 2, with exon 2 coding for the N-terminal domain (NTD), exons 3 and 4 coding for the DNA binding domain (DBD) and exons 5 until the first part of exon 9 coding for the ligand binding domain (LBD). Exon 1 is located in the promoter region and is not translated. Two distinct exons 1 have been identified, exon 1α and exon 1β (in rats also an exon 1γ has been distinguished) each having its own promoter, P1 and P2, resulting in the distinct mRNA transcripts MRα and MRβ (Zennaro et al., 1995). The two promoter regions contain distinct regulatory sequences, providing the ability for context- and tissue-specific gene expression (Zennaro et al., 1996). Indeed, MRα and MRβ are differentially regulated by hormones *in vitro*; while P2 is induced by dexamethasone and aldosterone, P1 is only induced by dexamethasone (Zennaro et al., 1996). The two *MR* splice variants MRα and MRβ are expressed at distinct levels in human peripheral tissues (Zennaro et al., 1997). No data exist on differential MRα vs. MRβ expression in human brain. However, rat studies indicate that expression of the 5' splice variants varies between the hippocampal subregions (Vazquez et al., 1998). Additional splice variants have been identified for the *MR* gene's coding region, but this is outside the scope of this thesis.

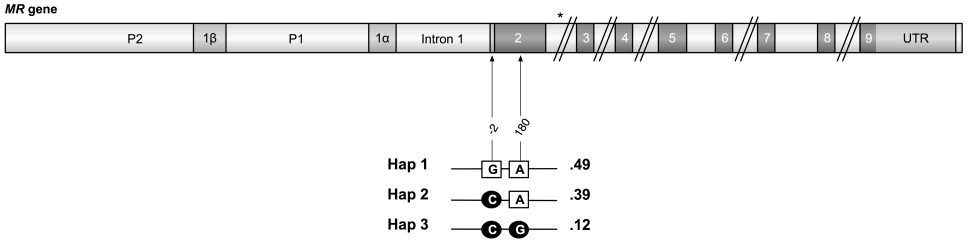


Figure 3 Schematic overview of the *MR* gene with its respective common and functional SNPs and haplotypes. Due to differential splicing only exon1a or exon1b is used during transcription, leading to two different mRNA variants, MRα or MRβ. Exons 2 to part of exon 9 are translated into protein (dark grey boxes). Exon1a and exon1b, the first 2 nucleotides of exon 2 and part of exon 9 are not translated (light grey boxes). Nucleotide differences as compared to the most frequent haplotype 1 are indicated (black ovals). Haplotype frequencies are based on genotypes of 50 anonymous blood DNA samples obtained from the general physician laboratory in Leiden (DeRijk et al., 2011). The SNPs in exon 2 are not related to SNPs more 3' in the *MR* gene as a recombination hotspot exists in intron 2 (asterisk). Abbreviations: P1, promoter 1; P2, promoter 2; UTR, untranslated region.

Functional single nucleotide polymorphisms in the mineralocorticoid receptor (*MR*) gene

The *MR* gene coding sequence has been thoroughly investigated in the search for SNPs, using 50 anonymous blood DNA samples obtained from the general physician laboratory in Leiden (DeRijk et al., 2011). Two common SNPs were identified, the *MR* -2G/C and I180V SNPs. With the help of functionality assays in cell lines it was found that the V-allele of the

I180V SNP results in a lower transactivation of target genes after binding its ligand cortisol (but not if aldosterone is used as a ligand (DeRijk et al., 2006)). On the other hand, the C-allele of the -2G/C SNP results in a higher protein expression and higher transactivational capacity (van Leeuwen et al., 2010a, 2010b). Together, the two SNPs result in three common haplotypes (heritable combinations of SNPs) with frequencies of .49; .39; .12 (**Figure 3**; the possible haplotype 4, -2G/180V, is very rare). Haplotype 2 is associated with the highest protein expression and transactivational capacity (van Leeuwen et al., 2011). Furthermore, the *MR* -2G/C and I180V SNPs are related to variability in activity of the sympathetic nervous system (DeRijk et al., 2006; Martinez et al., 2009; van Leeuwen et al., 2010b, 2011) and HPA reactivity (DeRijk et al., 2006; Kuningas et al., 2007; van Leeuwen et al., 2010a, 2011), although the results are sometimes somewhat inconsistent (Ising et al., 2008; Tobin et al., 2008). The -2 C-allele is associated with lower cortisol, while the 180 V-allele is associated with higher cortisol levels, particularly in combination with a 'challenge' (e.g. dexamethasone treatment, psychosocial stressor). The consensus is that a higher-active MR variant (demonstrated *in vitro*) is associated with a lower non-stress HPA activity. On the other hand, after a psychosocial stressor, subjects homozygous for haplotype 2 show the highest HPA- and autonomic response, suggesting that a higher-active *MR* variant makes an individual more dynamic during coping with a psychosocial challenge (van Leeuwen et al., 2011). With respect to cognition, this same study reported on *MR* haplotype-related variability in perceived chronic stress at work among teachers; compared to haplotype 1 and 3, haplotype 2 carriers experienced their work conditions as less stressful (van Leeuwen et al., 2011). Finally, recently it was found that healthy *MR* 180 V-allele carriers seem to be less able to modulate behavior as a function of reward after an acute stressor (Bogdan et al., 2010). In this thesis it was assessed whether the -2G/C and I180V SNPs are linked to other SNPs in the *MR* gene 5' untranslated region (5' UTR) and whether these other SNPs modulate the associations found with the exon 2 SNPs (see also *Experimental procedure of a candidate gene study*).

Sex differences

The associations found with the functional *MR* SNPs often differ among the sexes. Sex differences are often found in neuroscience, including molecular and genetic neuroscience (Jazin and Cahill, 2010). With respect to the stress system, men and women show differences in HPA activity during non-stress and stressful conditions (Kudielka and Kirschbaum, 2005), while glucocorticoids regulate specific gene pathways in a sex-dependent manner (Duma et al., 2010). In addition, men and women differ in their personality (Schmitt et al., 2008) and also differ in their risk of psychopathology, with women having a two times higher risk of depression compared to men (Bijl et al., 1998). The reason why MR activity often differs among the sexes may be in part due to the fact that sex steroids modulate MR mRNA and/or protein expression, resulting in a higher central MR expression in females compared to males (Carey et al., 1995; Castren et al., 1995; Turner, 1997; Watzka et al., 2000). In addition, various progesterone metabolites are able to bind the MR, with potential agonistic and antagonist effects (Quinkler et al., 2002). Furthermore, important to note here is that the common *MR* haplotypes shown in **Figure 3** confer differences in MR activity with varying ligand availability (van Leeuwen et al., 2011). Together this may result in sex-dependent stress-reactivity.

The associations found with the common and functional -2G/C and I180V SNPs often differ among the sexes.

1.5 Experimental procedure of a candidate gene study

A large number of genetic association studies among healthy or diseased groups of subjects are done without any prior knowledge on the functionality of the genetic variants on a molecular level. In many cases this may result in associations with SNPs of which it is not known whether they are the real causative SNPs, as SNPs may be linked to each other (see also *Testing functionality of gene variants in cell lines*). Here, genetic association studies were combined with functionality assays in cell lines. When SNPs are identified to have functional effects on a molecular level, it is more likely that they make a difference *in vivo*. Furthermore, their function *in vitro* can provide an *a priori* hypothesis with respect to the phenotype of interest. Moreover, their *in vitro* function can possibly provide important information for understanding the underlying mechanisms and for future therapy. The methods that were used in the present research are explained shortly in the following sections. It is important to note that often SNPs do not exist independently of other genetic variations, including SNPs. SNPs can be inherited together as a haplotype (combinations of SNPs located on a single chromosome) when they are in linkage disequilibrium (LD).* Combinations of SNPs can have distinct effects on gene functioning compared to the single SNPs, or the effect of one SNP can be masked by another SNP (van Rossum et al., 2004, Hummelshoj et al., 2006).

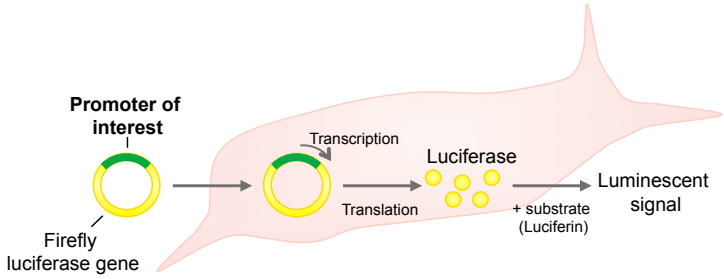
Testing functionality of gene variants in cell lines

Here it was assessed whether the -2G/C and I180V SNPs are linked to SNPs more 5' of the *MR* gene. The promoter region of a gene potentially contains many regulatory DNA sequences, such as binding sites for transcription factors (TFs). Next to the transcription initiation complex and other co-factors, TFs regulate gene transcription and mRNA expression. SNPs can affect the consensus sequence of a TF binding site and hereby influence gene transcription.

* SNPs can be inherited independently (Mendelian inheritance). However, due to linkage disequilibrium (LD) SNPs can also be inherited together. The level of LD between SNPs in a population can be calculated. Common measures that are used to indicate LD are *D'* and r^2. Scores for *D'* and r^2 can range from 0.0 to 1.0, with 1.0 meaning 100% LD; the genotype for SNP 1 perfectly predicts the genotype for SNP 2. The two LD measures have slightly different meanings. A *D'* of 1.0 means for example that allele A of SNP 1 always occurs together with allele A of SNP 2, but it is not necessarily true that allele B of SNP 1 always occurs together with allele B of SNP 2. This can occur when the allele frequencies of SNPs 1 and 2 are not the same. *D'* is a measure of recombination and is used to reconstruct haplotypes. An r^2 of 1.0 also means for example that allele A of SNP 1 always occurs together with allele A of SNP 2, but in addition allele B of SNP 1 always occurs together with allele B of SNP 2. The allele frequencies of SNPs 1 and 2 have to be the same (Zondervan and Cardon, 2004). R^2 indicates the correlation between two SNPs and is used to select tagging SNPs. Tagging SNPs are SNPs that can be genotyped in order to capture a complete haplotype structure (allele combination) along a chromosome, taking away the need to genotype all SNPs that constitute the haplotype. Tagging SNPs are selected based on their ability to distinguish one haplotype from another.

One way to test the functionality of SNPs is by performing DNA construct reporter assays in cell lines (**Box 2**). Multiple DNA reporter constructs are prepared, each containing a different allele of the gene variant of interest. The distinct constructs can subsequently be transfected in cells and compared for differences in activity.

Box 2 Testing effects of SNPs on promoter activity



Effects of SNPs on promoter activity can be tested by a reporter assay. Reporter constructs are prepared in which the promoter region is put in front of a so-called reporter gene. The firefly luciferase reporter gene encodes a protein that results in a luminescent signal after addition of its substrate beetle luciferin. Multiple DNA reporter constructs are prepared, each containing a different allele of the gene variant of interest. The distinct constructs are transfected in cells in separate culture dishes and depending on their activity the distinct promoters will start to regulate luciferin expression. All cells are co-transfected with a second reporter construct encoding *Renilla* luciferase (not shown in the figure for simplicity reasons), which after addition of its substrate coelenterate-luciferin results in a different luminescent signal. This is used in order to normalize for differences in transfection efficiency or cell death. After an incubation period (generally 1 or 2 days) the cells are lysed and the first substrate is added, generating the luciferase luminescent signal that can be measured in a luminometer. Then the second substrate is added to measure the *Renilla* luminescent signal.

Genetic association studies in the population

Because extensive candidate gene studies in the field of psychiatry have not resulted in many replicable findings, nowadays more and more researchers make use of genome-wide association studies (GWAs) and larger numbers of subjects (including thousands of patients and controls) in order to identify novel causal genes (Sullivan et al., 2009). Others question whether the genes identified with GWAs will be of any biological relevance (Abbott, 2008). The present research focuses on a single candidate gene. Three decades of animal studies indicate that the MR is abundantly expressed in the limbic brain and mediates corticosterone action on appraisal, behavioral flexibility and emotions. Recent work by van Leeuwen and DeRijk and the present research aims to translate those findings to the human situation. Association studies are performed with SNPs along the *MR* gene that are functional on a molecular level. The identification of different biologically relevant polymorphisms in the same gene can also be seen as a replication of a genetic association with a specific phenotype (McClellan and King, 2010). Furthermore, besides the single SNPs, specifically

the haplotypes are tested for their association with psychological phenotypes (emotional reactivity and personality) and physiological phenotypes (neuroendocrine activity) in healthy and/or patient groups.

1.6 Objective of this thesis

As outlined in the previous paragraphs, there are several indications that MR functioning and its dynamic regulation is central to physiological and psychological health. The **objective** of this thesis is to assess the influence of functional *MR* gene variants on psychological functioning in individuals selected from the general population and on the risk of depression.

The specific aims are:

- To assess the distribution of total MR mRNA and its splice variants in the *postmortem* human limbic brain and to test whether there are differences in MR vs. GR expression between depressed patients and non-depressed controls.

- To analyze the *MR* promoter region for the occurrence of single nucleotide polymorphisms (SNPs) and haplotypes and to test their influence on MR expression.

- To test the association between the common and functional *MR* gene variants with personality characteristics and cognitive reactivity in subjects selected from the general population.

- To test the association between the common and functional *MR* gene variants with neuroendocrine activity in subjects with diagnosis of depression.

- To test the association between the common and functional *MR* gene variants and risk of depression.

Outline of this thesis

Expression and distribution of total MR mRNA and its splice variants in the human limbic brain was assessed in *postmortem* brain tissue with the help of quantitative PCR. **Chapter 2** describes the methods and results based on tissue from five key brain regions obtained from six non-depressed subjects. Total MR vs. total GR mRNA expression was compared between the five brain regions and the patterns were compared with brain tissue obtained from six depressed subjects.

The positions, frequencies and functionality of SNPs in the *MR* gene promoter region were assessed, described in **Chapter 3**. To search for SNPs, almost 4000 basepairs of the *MR* gene promoter region were sequenced based on 50 anonymous blood DNA samples

obtained from the general physician laboratory in Leiden. Haplotypes (combinations of SNPs that occur together) were reconstructed and tested for their functionality in cultured cells.

Chapter 4 describes the results on an association between the three most common and functional *MR* haplotypes with the positive personality trait dispositional optimism among participants of the Arnhem Elderly Study. Dispositional optimism is a psychological trait that predicts lower risk of depression in later life. To verify the influence of the GR, several common *GR* gene variants were taken along.

In **Chapter 5** the common and functional *MR* haplotypes were tested for their association with cognitive reactivity to sad mood in students (measured with the Leiden Index of Depression Sensitivity-revised, LEIDS-R). Cognitive reactivity to sad mood predicts history of depression and depression relapse. The main goal was to test the association with thoughts of hopelessness, as opposed to optimism.

Chapter 6 describes the relation between the common and functional *MR* SNPs and haplotypes and neuroendocrine activity in depressed participants of the Netherlands Study of Depression and Anxiety (NESDA).

Chapter 7 describes the results of the association study between the *MR* gene variants and diagnosis of depression, using depressed subjects of the NESDA study and a large group of subjects without depression as a control group, selected from the Netherlands Twin Registry.

In **Chapter 8** the effects of *MR* gene variants on a dynamic regulation and functioning of the MR are discussed with regard to their possible implications for resilience or vulnerability to depression and for future therapeutic interventions.