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

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General Discussion

Contents

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

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

1. Location of genetic variation in the MR

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

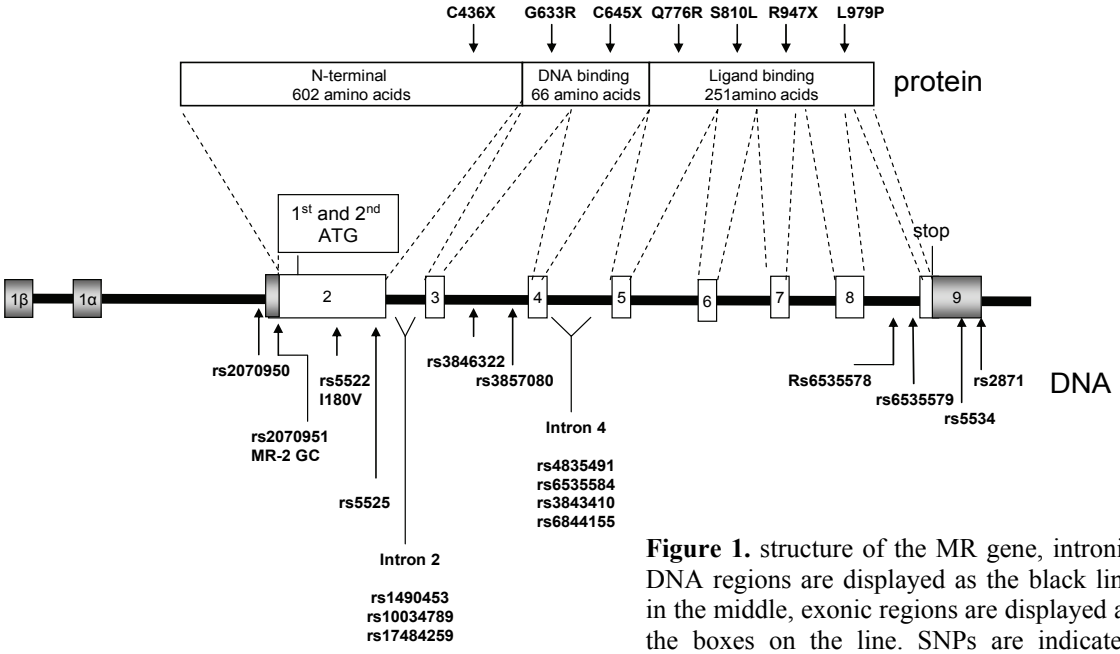

Figure 1. structure of the MR gene, intronic DNA regions are displayed as the black line in the middle, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA, while the protein structure and mutations are displayed above

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

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

2. Haplotypes in the MR

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

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

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

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

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

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

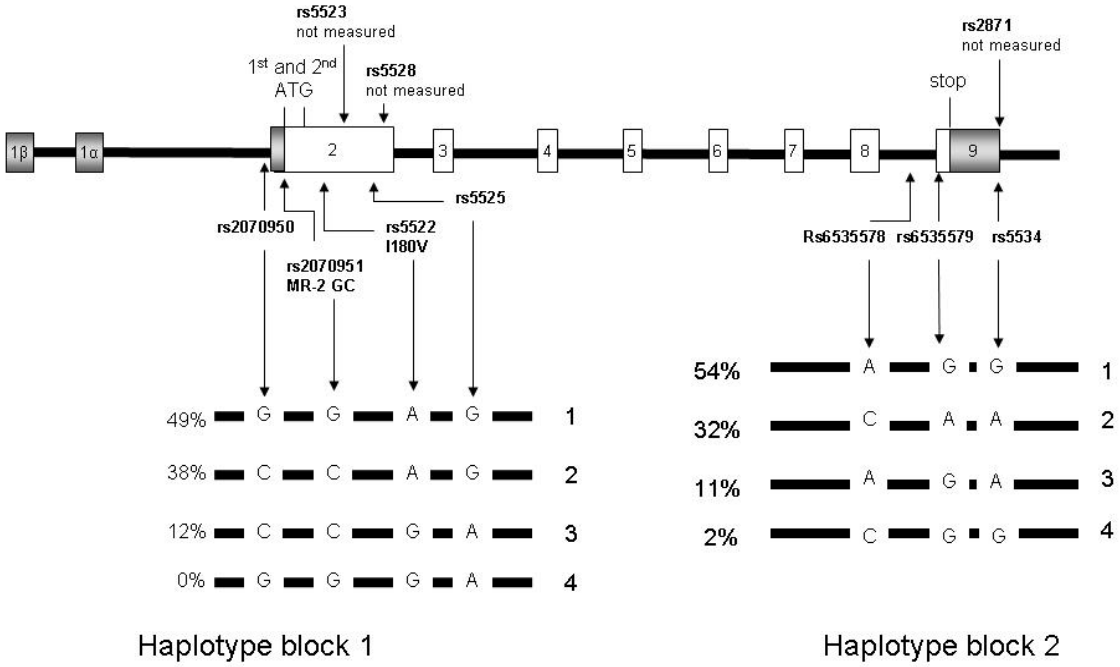

Figure 2. The hMR gene with SNPs and the two haplotype blocks formed by these SNPs. Intronic DNA regions are displayed as the black line on top, exonic regions are displayed as the boxes on the line. SNPs are indicated below the DNA and the haplotypes formed by the SNPs are indicated under the SNPs.

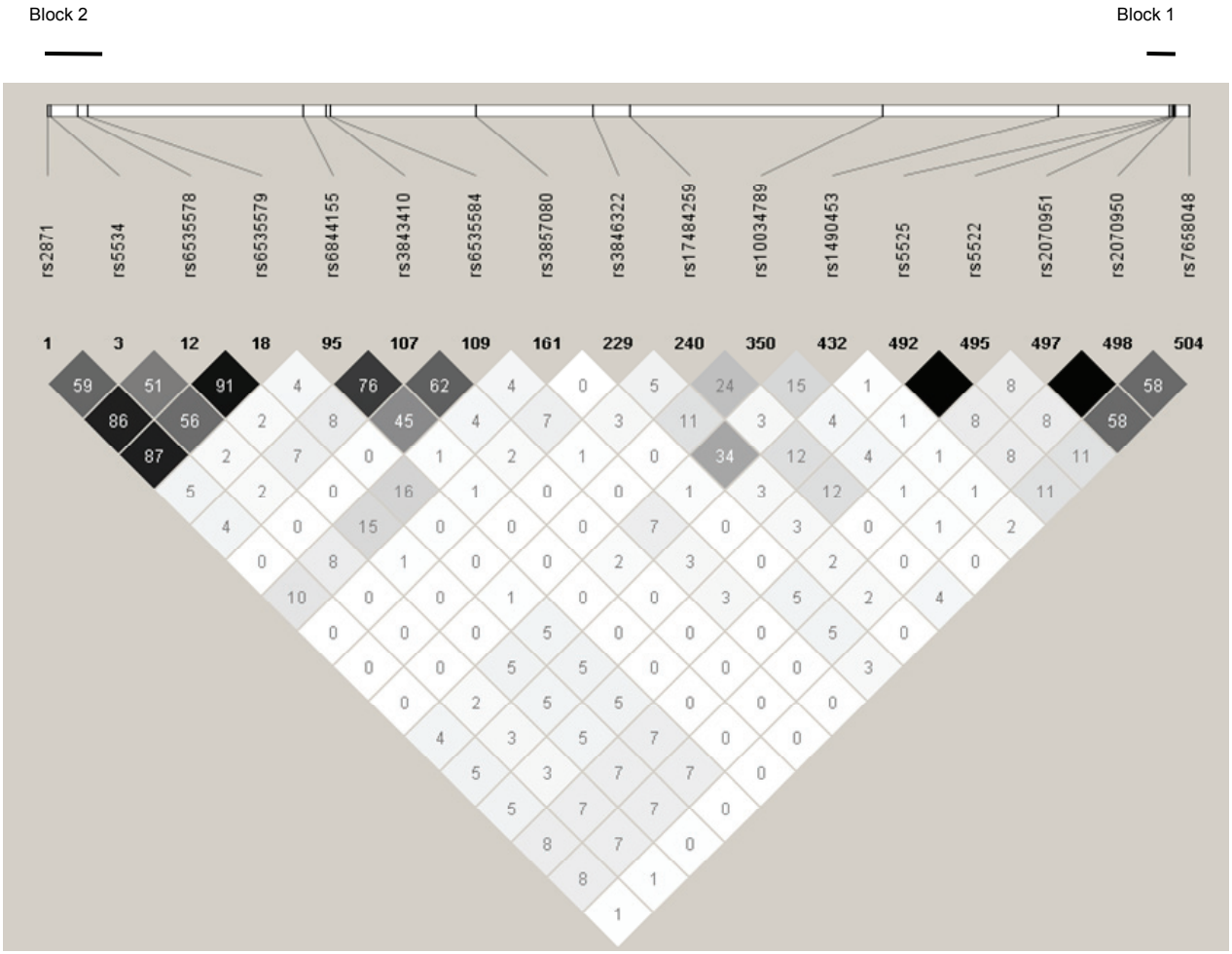

Figure 3. Linkage disequilibrium between the MR SNPs previously reported and reported in this manuscript. Black squares indicate $r^2= 1$ and shades of grey indicate $r^2<1$

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

3. Predicting functionality of SNPs and haplotypes

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

Table 1. Computer programs used for predicting functionality of SNPs

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

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

Table 2. Putative function of SNPs in the MR

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

4. Testing SNPs and haplotypes *in vitro*

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

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

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

4.1. MR-2G/C

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

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

4.2. MRI180V

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

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

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

4.3. Haplotypes

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

4.4. Conclusion testing SNPs and haplotypes *in vitro*

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

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

5. Association studies MR

5.1. Overview associations MR

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

Table 4. Associations with MR SNPs and MR haplotypes

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

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

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

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

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

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

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

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

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

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

5.3. Associations related to the Renin Angiotensin system

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

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

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

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

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

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

5.4. Effect of gender

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

5.5. Interaction with other genes

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

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

5.6. Associations MR SNPs with unknown function

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

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

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

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

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

6. Future perspectives

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

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

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

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

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