Mineralocorticoid receptor in human brain : a key player in resilience

Klok, M.D.

Chapter 3

Functional haplotypes in the human mineralocorticoid receptor gene promoter region

Melanie D. Klok, Nienke van Leeuwen, Frans G. Zitman,
E. Ronald de Kloet, Roel H. de Rijk

Accepted for publication in Translational Psychiatry together with Chapters 4, 5 and 7.

Abstract

Background: Mineralocorticoid receptor (*MR*) gene polymorphisms have been associated with variability in MR translation and transactivation and in neuroendocrine and autonomic regulation. No reports exist on polymorphisms in the *MR* gene promoter region, which potentially influence *MR* transcription, splicing and context-dependent MR expression. Here we tested whether the two known functional *MR* single nucleotide polymorphisms (SNPs; *MR* -2G/C and I180V) in exon 2 are in linkage disequilibrium (LD) with SNPs in the gene's promoter region.
Methods: A sequence of almost 4 kilobases of the *MR* gene promoter region was analyzed for the occurrence of SNPs and haplotypes by sequencing the DNA isolated from 50 anonymous blood samples. Functionality of the common haplotypes was tested by reporter assays.
Results: Three common haplotypes were identified which were highly linked to the functional *MR* -2G/C and I180V SNPs. In comparison to the haplotypes 1 (linked to -2G/180I; freq. .50) and 3 (linked to -2C/180V; freq. .09), haplotype 2 (linked to -2C/180I; freq. .41) resulted in 1.4 or 2.2 times higher gene transcription after transfection in human neuroblastoma cells, respectively.
Conclusions: Together with previous work the data show that haplotype 2 results in higher gene transcription, translation and transactivation. We propose that the common and functional 5' *MR* haplotypes might relate to significant variability in MR expression in the brain, with possible consequences for its centrally regulated functions.

Key words: mineralocorticoid receptor, promoter, single nucleotide polymorphism (SNP), haplotype, transcription

Introduction

The mineralocorticoid receptor (MR) is originally known for binding the mineralocorticoid aldosterone in the kidney, hereby regulating salt- and water balance (Funder, 2005). In the brain the MR binds also the hormone cortisol, the end product of the hypothalamic-pituitary-adrenal (HPA) axis. This is because the brain generally lacks the cortisol-degrading enzyme 11β-hydrosteroid dehydrogenase type 2 (11βHSD2) present in the kidney (Edwards et al., 1988; Funder et al., 1988). Since the glucocorticoid hormone circulates in a 100- to 1000-fold excess over aldosterone, the brain *sees* therefore predominantly cortisol. In the brain, together with the glucocorticoid receptor (GR), the MR mediates in a complementary fashion cortisol effects on neuroendocrine regulation, behavior and cognition in both animals and humans. Aberrant corticosteroid receptor signaling can have considerable implications for the body's homeostatic regulations (De Kloet et al., 1998).

The *MR* gene 5' untranslated region (5' UTR) contains multiple exon 1s. In humans two exon 1s have been identified thus far, exon 1α and exon 1β (Zennaro et al., 1995), but the possibility exists for additional exon 1s, as in rat three exon 1s have been identified; exon 1a, 1b and 1g (Kwak et al., 1993). The presence of multiple promoters (P1 and P2) establishes the ability for alternative splicing and a wide capacity for transcriptional regulation (Zennaro et al., 1995, 1996). Indeed, the MR transcripts and protein are expressed in a time-, context- and tissue-dependent manner (Zennaro et al., 1997; Vazquez et al., 1998; Gesing et al., 2001; Pryce, 2008; Kang et al., 2009). In cell lines steroids differentially regulate the distinct promoters P1 and P2; both are induced by dexamethasone (a synthetic glucocorticoid), while only P2 is also responsive to aldosterone (Zennaro et al., 1996). Importantly, the highly dynamic MR expression has profound implications for the effects of corticosterone on HPA activity, behavior, neuronal survival and ageing (van Eekelen et al., 1991; Gesing et al., 2001; Macleod et al., 2003; Schmidt et al., 2004; Topic et al., 2008). Together the data indicate that the *MR* 5' UTR is an important regulatory region establishing the opportunity to dynamically regulate MR protein if needed.

Besides splice variants, multiple *MR* single nucleotide polymorphisms (SNPs) exist, of which the *MR* -2G/C and I180V have been most extensively studied. The two SNPs located in exon 2 and their respective haplotypes influence MR translation and/or the MR's transactivational capacity in cell lines (DeRijk et al., 2006; van Leeuwen et al., 2010a, 2010b, 2011). In addition, both SNPs were found to associate with inter-individual variability in neuroendocrine and/or autonomic activity (DeRijk et al., 2006; Kuningas et al., 2007; Martinez et al., 2009; van Leeuwen et al., 2010a, 2010b, 2011). Moreover, one report suggested an association between the I180V SNP and symptoms of geriatric depression (Kuningas et al., 2007), while more recent studies suggest the *MR* SNPs to relate to variability in perceived chronic stress (van Leeuwen et al., 2011) or behavior during a reward task (Bogdan et al., 2010). Considering these wide effects of the *MR* -2 G/C and I180V SNPs it is important to know that additional SNPs exist more 5' and 3' in the *MR* gene. However, due to recombination the exon 2 SNPs are not linked to the SNPs 3' of intron 2 (DeRijk et al., 2011). On the other hand, online databases (HapMap; (http://hapmap.ncbi.nlm.nih.gov) show that the *MR* promoter region contains multiple SNPs,

which potentially influence *MR* transcription and its dynamic expression. Still, limited data are available on SNP frequencies and haplotypes in the *MR* promoter region. We aimed to assess the positions and frequencies of SNPs and haplotypes in a stretch of almost 4 kilobases (kb) of the human *MR* promoter region, including P1, P2, exon 1a and exon 1b and we have determined their functionality.

Methods

PCR and sequencing of the *MR* gene promoter region

Previously, 50 anonymous blood samples were obtained from the general physician laboratory in Leiden. DNA was isolated and the *MR* gene's coding sequence was analyzed for the occurrence of SNPs, including the -2G/C and I180V SNPs, as described by DeRijk *et al.* (DeRijk et al., 2011). In the present study, a region of almost 4 kilobases (kb; 3870 basepairs) of the 5' UTR was amplified and the sequence was analyzed for the occurrence of SNPs. To that end multiple PCR reactions were performed. Because of the high GC content of the *MR* promoter region a PCR kit specifically designed for GC-rich regions was used, the GC-RICH PCR system (Roche Diagnostics, Mannheim, Germany). PCR-reactions were performed in a total volume of 25 μL and contained 1 x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen Life Technologies, Breda, The Netherlands), 0.3 μM forward and reverse primer (Isogen Life Science, Maarssen, The Netherlands), 0.5 M solution 3, 1 unit of enzyme and 50 ng of DNA. PCR was performed in a PTC-200 (MJ Research Inc, Watertown, MA, USA) using the following conditions: an initial denaturation step for 3 min at 95°C, followed by 35 cycles of 60 sec (or 90 sec for products larger than 1 kb) at 95°C, 30 sec at the specific annealing temperature and 60 sec (or 90 sec for products larger than 1 kb) at 72°C and a final extension step of 7 min at 72°C. The PCR products were purified with columns (Machery-Nagel, Düren, Germany) followed by sequencing PCR. Sequencing PCR was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Niewerkerk a/d Ijssel, The Netherlands) according to the manufacturers instructions and products were analyzed on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems). The 4 kb sequence of the *MR* promoter region was initially analyzed using seven different primer sets. Sequencing PCR was performed using the same forward and/or reverse primer. Nested primers were used where sequences were unclear. In case of detection of a novel SNP (sequence compared to NCBI36:4:149582036:149585904, Ensembl, January 2007) a second set of primers was used to validate this. The sequences of the different primer sets, specific annealing temperatures and product sizes are listed in **Table 1**.

Prediction of SNP effects on *MR* transcription

Based on the literature we verified whether any of the common *MR* 5' UTR SNPs were located in previously identified transcription factor (TF) binding sites (Zennaro et al., 1996). Moreover, common SNPs were analyzed for allele-specific binding of matrix conservation based predicted glucocorticoid responsive elements (GREs) or TFs with the help of the TRANSFAC 4.0 database (Matys et al., 2003) (using the tool AliBaba 2.1, available online at http://www.gene-regulation.com/pub/ programs.html, September 2010, minimal matrix

conservation set at 75%) and the JASPAR database (Sandelin et al., 2004) (JASPAR CORE homo sapiens database, tool available online at http://jaspar.genereg.net/, September 2010, relative profile score threshold set at 75%). Finally, effects of the SNPs on splicing of the MR transcripts were explored (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al., 1997).

Table 1 Locations, sequences, annealing temperatures and product sizes of primers used for SNP analysis of 4 kb of the human *MR* promoter region

Primer location	Sequence	Ann. Temp.	Product size
Promoter 2	FWD: 5'-CGA GGA GCA GGA AAA GAA AA-3'	56	670
Promoter 2	REV: 5'-GGT GAG GAT GGA GAG GAT GA-3'		
Promoter 2	FWD: 5'-GAT CCT CCT GCC GGA CTT-3'	58	610
Promoter 1	REV: 5'-CCC TGG ATC TCA GCT TCT TG-3'		
Promoter 1	FWD: 5'-CCG CCT CTT GTA GGG TAA CA-3'	58	452
Promoter 1	REV: 5'-CCC TGG ATC TCA GCT TCT TG-3'		
Promoter 1	FWD: 5'-GAC AGT CAC TTT GCG CTG AC-3'	56	1010
Promoter 1	REV: 5'-AAT TTC GGT TTC CCT CCA AC-3'		
Promoter 1	REV: 5'-GGA ACT CCC TGG AGA TAG GG-3'		
Promoter 1	FWD: 5'-GGC ATT AGA GTC TGG GGT CA-3'		
Promoter 1	FWD: 5'-GGG GGA CCA GAT TTA GGT GT-3'	58	868
Promoter 1	REV: 5'-CAC CCT GCT CTC CTT CTG AC-3'		
Promoter 1	*FWD: 5'-GGC ATT AGA GTC TGG GGT CA-3'*	57	696
Promoter 1	*REV: 5'-AAG AAG TGG CAG GGT CAA GA-3'*		
Promoter 1	FWD: 5'-AGA CAG TGG AAA GGG GCT G-3'	57	1322
Intron 1	REV: 5'-TCC TTC AAC TGC CCT TATG C-3'		
Promoter 1	*FWD: 5'-AGA CAG TGG AAA GGG GCT G-3'*	59	895
Promoter 1	*REV: 5'-TCT CTC GCC GTC TAC CTG TT-3'*		
Promoter 1	*FWD: 5'-CAG GGT GGA CGT AAG CAA GT-3'*	59	662
Promoter 1	*REV: 5'-TCT CTC GCC GTC TAC CTG TT-3'*		
Exon1α	*FWD: 5'-AAC AGG TAG ACG GCG AGA GA-3'*	57	548
Exon1α	*REV: 5'-AGG AAG CGT AGC CTG TCT CA-3'*		
Intron 1	FWD: 5'-TAC CAC CCT TCC CTT TAC CC-3'	58	469
Intron 1	REV: 5'-GGT TTC AAA AGC TCG TCT GC-3'		
Promoter 2	FWD: 5'-CG**G GTA CC**C GAG GAG CAG GAA AAG AAA A-3'	62	3886
Intron 1	REV: 5'-CG**A GAT CT**G GTT TCA AAA GCT CGT CTG C-3'		

Notes: A 4kb sequence of the *MR* promoter region was analyzed using initially seven different primer sets. Sequencing PCR was performed using the same forward and/or reverse primer. Additional sequencing primers (underlined) were used in case the sequence was not clear along the complete amplification product. Additional PCR primer sets (italic) were used to verify the results of the first set of primers in case novel SNPs were detected. The last set of primers was used for amplification of the complete 4 kb *MR* promoter region and the product was used for reporter plasmid construction. Sequences for restriction factor binding sites are indicated in bold text.
Abbreviations: FWD, forward primer; REV, reverse primer; Ann. Temp., annealing temperature.

Construction of reporter plasmids

For the construction of reporter plasmids containing the *MR* promoter region with haplotype 1, 2, or 3, the sequence of 3870 basepairs was amplified using DNA of a subject that was identified as carrying one of these haplotypes. To the forward primer eight nucleotides were added at the 5' end including six nucleotides encoding a restriction site for the enzyme *Acc65*I, while to the reverse primer eight nucleotides were added at the 3' end including six nucleotides encoding a restriction site for the enzyme *Bgl*II (**Table 1**). PCR-reactions

contained 1 x buffer (GC-RICH PCR system, Roche), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM forward and reverse primer, 0.5 M solution 3, 1 unit of enzyme and 50 ng of DNA in a total volume of 25 μL. PCR was performed using the following conditions: an initial denaturation step for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 sec at 62°C and 3 min at 68°C and a final extension step of 7 min at 72°C. The PCR products were purified with columns, cloned into the intermediate vector pGEM-T Easy (Promega, Leiden, The Netherlands) and sequenced to confirm the nucleotide sequence. Subsequently, the *MR* promoter sequences were cut out of the pGEM-T Easy Vector with the restriction enzymes *Acc65*I and *Bgl*II and ligated (T4 DNA Ligase, Promega) into the pGL3-Basic luciferase reporter vector (Promega), which was cut open with the same restriction enzymes. The constructs were transformed into JM109 competent cells (Promega), purified from the bacterial cultures using the PureYield purification system (Promega) and sequenced to confirm the nucleotide sequence. Two separate bacterial cultures and plasmid DNA isolates were prepared for each of the three haplotype-firefly luciferase constructs on two distinct days.

Cell culture, transient transfection and luciferase assays

Human neuroblastoma cells BE(2)-M17 (Health Protection Agency Culture Collections, Cat. No. 95011816) were kept at 70-80% confluence in GIBCO Opti-MEM I Reduced Serum Medium with L-Glutamine (Invitrogen Life Technologies, Breda, The Netherlands) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO) and 1% penicillin/streptomycin (Pen/Strep, GIBCO). One day before transfection 50.000 cells were seeded in 24-well plates in growth medium without Pen/Strep. For transfection 1 μL of Lipofectamine 2000 (Invitrogen) was used to transfect 200 ng of the haplotype-firefly luciferase construct 1, 2 or 3, together with 10 ng of pGL4.74[*hRluc*/TK] *Renilla* luciferase reporter vector (Promega). In separate wells, 100 ng of pGL3-Basic or pGL3-Control vector (Promega) were transfected, functioning as background measurement or positive control, respectively. Four hours after transfection, culture medium was removed and fresh growth medium with Pen/Strep was applied to the cells. Each construct was transfected in six separate wells. After 48 hours of incubation cells were lysed in 100 μL of Passive Lysis Buffer (PLB, Promega). Firefly and *Renilla* luminescent activity was assessed sequentially in 25 μL of lysate after addition of 25 μL of Luciferase Assay Buffer II followed by addition of 25 μL of Stop & Glo Buffer (Promega). Luminescent signals were measured for 10 sec in a Berthold CentroXS^3 LB 960 Microplate Luminometer (Berthold, Bad Wildbad, Germany). Experiments were performed three times on separate days for each of the two sets of plasmid DNA isolates. Relative light units (RLUs) were calculated by dividing the firefly luminescent signals (promoter activity) by the corresponding *Renilla* luminescent signals in order to correct for variability in transfection efficiency or cell death.

Statistical analysis

Allele frequencies for the different SNPs were tested for Hardy-Weinberg equilibrium (HWE) using HaploView (version 4.1 for Mac OS X; available online at http://www.broadinstitute.org/mpg/haploview (Barrett et al., 2005). In addition, HaploView was used to assess inter-marker linkage disequilibrium (LD) scores (expressed as *D'* and r^2) between the *MR* SNPs and to reconstruct haplotypes. A one-way analysis of variance (ANOVA) was conducted

followed by a *post hoc* Bonferroni multiple comparison test to compare promoter activities between the three constructs containing haplotype 1, 2 or 3 using SPSS, version 16.0 for Mac OSX (SPSS Inc., Chicago, IL, USA).

Results

MR SNP and haplotype frequencies and/or structure

Sixteen SNPs were detected along the 4 kb *MR* promoter region. As of June 2011, 3 SNPs were still novel (GRCh37:4:149362585:149366454, Ensembl; SNP nr. 1, 4, 6), while the 5 other SNPs now have a rs-number. All allele frequencies of the *MR* SNPs were in HWE (p> .10). For an overview of individual SNP genotype frequencies see **Table 2**.

Table 2 Allele- and genotype frequencies of sixteen SNPs located along 4 kb of the human *MR* promoter region

SNP	SNP number	Location	Alleles n / allele / frequency						Genotypes n / genotype / frequency									HWE (p)
1	Novel SNP 1 G/C	149366331	103	G	.99	1	C	.01	51	G/G	.98	1	G/C	.02	0	C/C	.00	1.0
2	**rs9992256 C/T**	149366293	**54**	**C**	**.52**	**50**	**T**	**.48**	**12**	**C/C**	**.23**	**30**	**C/T**	**.58**	**10**	**T/T**	**.01**	**.44**
3	**rs62332389 C/T**	149366170	**62**	**C**	**.60**	**42**	**T**	**.40**	**16**	**C/C**	**.31**	**30**	**C/T**	**.58**	**6**	**T/T**	**.11**	**.28**
4	Novel SNP 2 G/A	149365962	102	G	.98	2	A	.02	50	G/G	.96	2	G/A	.04	0	A/A	.00	1.0
5	**rs5520 G/C**	149365909	**62**	**G**	**.60**	**42**	**C**	**.40**	**16**	**G/G**	**.31**	**30**	**G/C**	**.58**	**6**	**C/C**	**.11**	**.28**
6	Novel SNP 3 C/A	149365846	103	C	.99	1	A	.01	51	C/C	.98	1	C/A	.02	0	A/A	.00	1.0
7	rs5521 T/C	149365769	101	T	.97	3	C	.03	49	T/T	.94	3	T/C	.06	0	C/C	.00	1.0
8	**rs3216799 -/CT**	**149365384**	**62**	**-**	**.60**	**42**	**CT**	**.40**	**16**	**-/-**	**.31**	**30**	**-/CT**	**.58**	**6**	**CT/CT**	**.11**	**.28**
9	**rs2248038 G/A**	149364999	**11**	**G**	**.11**	**93**	**A**	**.89**	**1**	**G/G**	**.02**	**9**	**G/A**	**.17**	**42**	**A/A**	**.81**	**.89**
10	**rs7671250 C/T**	149364780	**11**	**C**	**.11**	**93**	**T**	**.89**	**1**	**C/C**	**.02**	**9**	**C/T**	**.17**	**42**	**T/T**	**.81**	**.89**
11	rs61760029 C/T	149364607	103	C	.99	1	T	.01	51	C/C	.98	1	C/T	.02	0	T/T	.00	1.0
12	rs61760027 G/A	149363959	100	G	.96	4	A	.04	49	G/G	.94	2	G/A	.04	1	A/A	.02	.12
13	rs61760025 A/T	149363827	103	A	.99	1	T	.01	51	A/A	.98	1	A/T	.02	0	T/T	.00	1.0
14	rs60660883 G/A	149363106	103	G	.99	1	A	.01	51	G/G	.98	1	G/A	.02	0	A/A	.00	1.0
15	**rs6814934 C/G**	149362869	**54**	**C**	**.52**	**50**	**G**	**.48**	**12**	**C/C**	**.23**	**30**	**C/G**	**.58**	**10**	**G/G**	**.19**	**.44**
16	**rs7658048 C/T**	149362744	**66**	**C**	**.63**	**38**	**T**	**.37**	**18**	**C/C**	**.34**	**30**	**C/T**	**.58**	**4**	**T/T**	**.08**	**.16**

Notes: Allele- and genotype frequencies are based on 50 anonymous DNA samples. All SNPs were in Hardy-Weinberg equilibrium (HWE; p> .10). The eight SNPs included in the three common 5' UTR haplotypes are indicated in bold text.

Reconstruction of *MR* haplotypes resulted in one haplotype bin that was highly linked to the previously described *MR* -2G/C and I180V SNPs (**Figures 1 and 2**). The inter-marker correlations (r^2, **Figure 1**) and *D'* LD values ranged from .00 to 1.00. Three haplotypes with a frequency of above .03 were identified, haplotype 1 (freq. .48), haplotype 2 (freq. .34) and haplotype 3 (freq. .07), while seven haplotypes were identified that had a frequency below .03. Together with genotyping results based on additional cohorts (~ 1100 subjects) we observed that indeed the other seven minor haplotypes had frequencies below .03.

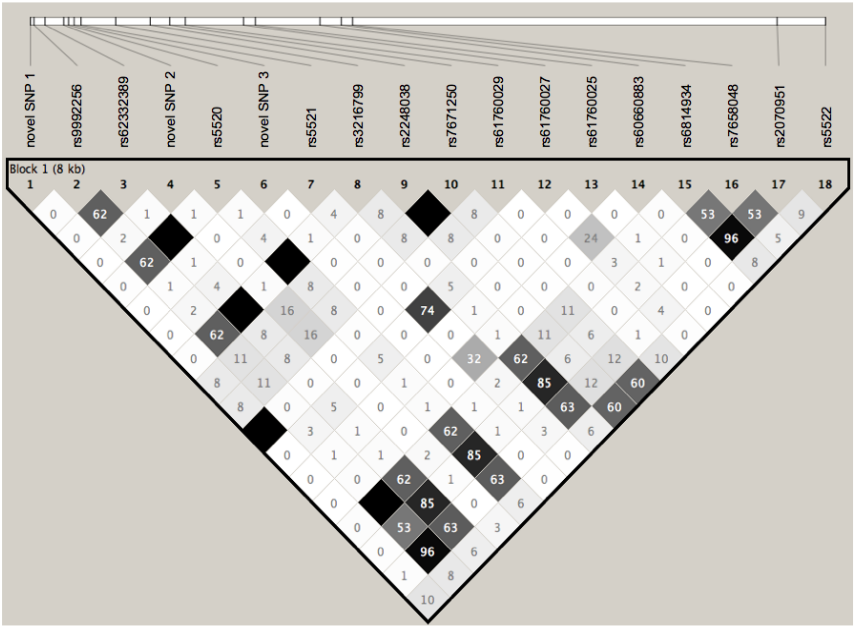

Figure 1 Overview of linkage disequilibrium (LD) along 4 kb of the human *MR* promoter region and including the -2G/C and I180V SNPs in exon 2. Linkage correlations (r^2) and relative positioning of the SNPs are indicated.

Pooling the low frequency haplotypes with the high frequency haplotypes (based on the -2G/C and I180V genotypes) resulted in final frequencies for the haplotypes 1 till 3 of respectively .50; .41 and .09, which differ in eight *MR* 5' UTR SNPs (**Figure 2**). These haplotype frequencies differ slightly from the results presented elsewhere (DeRijk et al., 2011) as the 50 blood DNA samples used here were slightly different. Haplotypes 1 till 3 were subsequently studied for functionality.

Predicted influence of *MR* promoter SNPs on transcription factor binding

The eight *MR* 5' UTR SNPs determining haplotype 1 till 3 were not located at previously described TF binding sites (Zennaro et al., 1996). However, *in silico* analysis using two different databases consistently predicted SNP 5 to affect the number of possibilities for Sp1 binding, while SNP 8 seems to influence HNF-1b (Hepatocyte Nuclear Factor 1b) binding (**Table 3**). Of interest is that the JASPAR database predicted SNP 9 to affect a GRE-consensus sequence by influencing binding of NR3C1 (or GR), while SNP 8 was predicted to be located 1 nucleotide next to a GRE (data not shown). The SNPs did not alter predicted splicing of the MR transcripts.

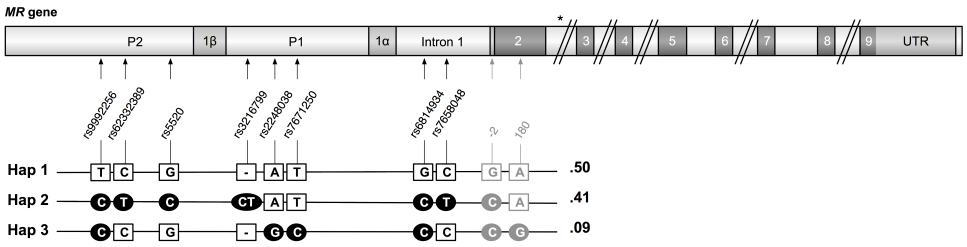

Figure 2 Schematic overview of the human *MR* gene with its respective 5' haplotypes and haplotype frequencies. The figure shows three haplotypes along a stretch of 4 kilobases of the 5' untranslated region (UTR) that were reconstructed based on the genotypes of 50 anonymous DNA samples and include eight SNPs. The positioning and relation with the -2G/C (rs2070951) and I180V (rs5522) SNPs (grey) are indicated, which tag the three common haplotypes. The haplotypes located in exon 2 and extending into the promoter region are not linked to common SNPs more 3' in the *MR* gene sequence, as a recombination hotspot exists in intron 2 (asterisk). The haplotype frequencies differ slightly from the results presented elsewhere (DeRijk et al., 2011) as the 50 blood DNA samples used here were slightly different.
Abbreviations: P1, promoter 1; P2, promoter 2; UTR, untranslated region.

Table 3 Predicted transcription factor binding at eight common *MR* 5' UTR SNP sites

SNP	Allele	TRANSFAC	JASPAR
2	T	*RAP1*; *IRF1*	*FOXI1*; *NFIC (1x)*
	C	*Sp1*; *ICSBP*	*NFIC (2x)*
3	C	*Egr-1*; *Sp1*; *ER*; *v-Myc*	MZF1_5-13; HIF1A::ARNT; NFKB1
	T	*Sp1 (2x)*	MZF1_5-13; HIF1A::ARNT; NFKB1
5	G	***Sp1 (3x)***	***SP1 (3x)***; *TFAP2A*; *HIF1A::ARNT*; ZNF354C
	C	***Sp1 (7x)***; *Ap-2*; *CACCC-bi*	***SP1 (6x)***; *TFAP2A (2x)*; ZNF354C
8	-	**…**	*BRCA1*; *IRF1*
	CT	***HNF-1***	***HNF1B***; *FOXC1*; *FOXD1*
9	A	…	*ELK1*; *ELK4*; *SPIB*; TFAP2A
	G	…	TFAP2A; *NR3C1*
10	T	*Erg-1*; Oct-1	SRF; HOXA5; *NFIL3*
	C	Oct-1	*NF-kappaB*; *REL (2x)*; SRF; *TFAP2A*; HOXA5; *GATA2*
15	G	Sp1	*RORA_2*; *MZF1_5-13*; *MZF1_1-4 (2x)*; ELK1
	C	Sp1	*MZF1_1-4*; ELK1
16	C	…	*NFIC*; *YY1*; ETS1
	T	…	*FOXC1*; *REL*; ETS1

Notes: *In silico* analysis of predicted TF binding was performed using the TRANSFAC and JASPAR databases. Allele-specific TF binding is indicated in italic text, consistently predicted allele-specific TF binding is indicated in italic, bold text. Note that according to the JASPAR database SNP 9 influences a GRE-consensus sequence as indicated by allele-specific binding of NR3C1 (italic and underlined).

MR promoter haplotype 2 results in higher gene transcription

Results of three independent experiments with two separate sets of plasmid isolates were highly similar. The *MR* promoter region was active under non-stimulating conditions with all three haplotypes, as the constructs resulted in a signal that was higher compared to the pGL3-Basic plasmid, while the positive control (pGL3-control) showed a signal that was

almost 25 times higher compared to the pGL3-Basic plasmid (**Figure 3**). Activities between the three plasmids differed significantly ($F(2, 15)= 27.98$; $p< .001$). Activity of the *MR* promoter region containing haplotype 2 was 1.4 times higher compared to the promoter region containing haplotype 1 ($p< .01$) and 2.2 times higher compared to the promoter region containing haplotype 3 ($p< .001$).

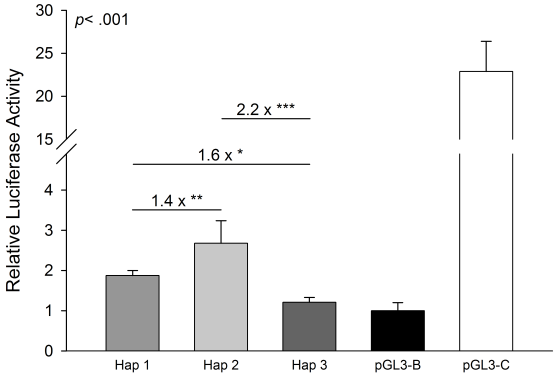

Figure 3 Activity of the human *MR* promoter region associated with haplotype 1, 2 or 3. The pGL3-Basic and pGL3-Control constructs were taken along as the negative and positive control, respectively. Results of three independent assays with two separate sets of plasmid isolates were highly similar. The figure shows the results of a representative assay. Data are firefly luminescent signals divided by the *Renilla* luminescent signals, hereby controlling for cell death and variability in transfection efficiency. Activities of the constructs containing haplotype 1 till 3 are shown relative to the activity of the pGL3-Basic plasmid, which activity was set to 1. Activities differed significantly between the three MR plasmids ($F(2, 15)= 27.98$; $p< .001$). Note the break in the y-axis. *$p< .05$; **$p< .01$; ***$p< .001$

Discussion

Screening of 4 kb of the human *MR* promoter region identified sixteen SNPs. Haplotype reconstruction resulted in three haplotypes with a frequency above .03 based on eight common 5' UTR SNPs, while seven additional minor haplotypes with a frequency below .03 were identified based on all sixteen SNPs. Transfection of distinct reporter constructs containing the common haplotype 1, 2, or 3 in a human neuroblastoma cell line showed that haplotype 2 resulted in a significantly higher transcriptional activity compared to haplotypes 1 and 3. Differences were found without external stimulation.

Differences in activity of a gene's promoter region due to SNPs can be expected. Indeed, *in silico* analysis of the 5' UTR using two different TF binding profile databases predicted multiple possibilities for allele-specific TF binding at the eight common SNP sites. Consistent results were found for SNP 5, which seems to affect the number of binding possibilities for Sp1 (specificity protein 1), a protein that is known to be ubiquitously involved in expression regulation of many different genes (Suske, 1999). In addition, SNP 8 potentially affects

HNF-1B (Hepatocyte Nuclear Factor 1b) binding, which is a transcription factor that is known to play an important role in regulation of target gene expression in amongst others the kidney (Igarashi et al., 2005). Interestingly, according to the JASPAR database SNP 9 affects a GRE-consensus sequence by affecting NR3C1 (or GR) binding. Moreover, the JASPAR database predicted that a GRE is located one nucleotide next to SNP 8 (data not shown). Allele-specific GR binding (or MR, as MR also binds to the same GREs) potentially results in inter-individual variability in context-dependent MR expression. However, the differential binding of NR3C1 predicted by the JASPAR database was not confirmed by the TRANSFAC database. *In vitro* assays, like electrophoretic mobility shift assays, are necessary to identify real allele-specific TF binding. None of the SNPs was predicted to affect splicing.

Importantly, the 5' UTR haplotypes 1 till 3 were highly linked to the functional -2G/C and I180V SNPs located in exon 2. Evidence is showing that these two SNPs are also modulating MR activity. The C-allele of the -2G/C SNP results in a higher translational activity, resulting in increased MR protein expression *in vitro* (van Leeuwen et al., 2010a, 2010b). On the other hand, the V-allele (or G nucleotide) of the I180V SNP results in a different MR primary structure (amino acid sequence) of its transactivation domain (exon 2); an isoleucine is changed into a valine (DeRijk et al., 2006). A different protein structure or a difference in protein expression potentially influences the MR's transactivational capacity and indeed, both the -2 C-allele and the 180 V-allele are related to higher or lower transactivation of a target gene after binding cortisol, respectively (DeRijk et al., 2006; van Leeuwen et al., 2010a, 2010b). Moreover, a recent report showed that the haplotype based on the -2 C-allele and the 180 I-allele (haplotype 2) results in higher MR protein expression and transactivational capacity (van Leeuwen et al., 2011). Together the data indicate that haplotype 2 results in higher *MR* transcriptional, translational and transactivational capacity. However, the present results cannot just be directly extrapolated to the *in vivo* situation. Additional research is required to determine whether these *MR* SNPs relate to differential MR expression in the brain (or in the periphery).

The present results are of putative relevance for inter-individual differences or changes in MR expression. MR expression is known to be tissue-specific (Zennaro et al., 1997; Pryce, 2008) and is highly dynamic; MR expression changes during development and aging and after physical or psychological stress (van Eekelen et al., 1991; Vazquez et al., 1998; Gesing et al., 2001; Macleod et al., 2003; Schmidt et al., 2004; Topic et al., 2008). Moreover, central MR expression is different in disorders like depression (Lopez et al., 1998; Xing et al., 2004; Wang et al., 2008) (**Chapter 2**), while multiple studies show that antidepressants induce hippocampal MR expression, possibly in part underlying their clinical effect in patients (Seckl and Fink, 1992; Barden et al., 1995; Bjartmar et al., 2000; Nickel et al., 2003; Zobel et al., 2004). These dynamic changes in MR expression may depend on the *MR* genotype, possibly underlying inter-individual differences in HPA activity, emotions and cognition.

Indeed, genetic association studies show that the functional *MR* -2G/C and I180V SNPs relate to inter-individual differences in physiological and psychological functioning, which to

a large extend may be due to the linked 5' UTR SNPs described here. The 180 V-allele was found to relate to a higher neuroendocrine and autonomic activity in response to a psychosocial stressor in healthy young men, while the -2 C-allele was found to relate to lower basal morning cortisol in elderly or to a lower cortisol awakening response (CAR) in dexamethasone pretreated healthy young men or in patients with a major depressive disorder (MDD) treated with a selective serotonin reuptake inhibitor (SSRI; **Chapter 6**) (DeRijk et al., 2006; Kuningas et al., 2007; van Leeuwen et al., 2010a). Moreover, haplotype 2 (determined by using the -2G/C and I180V SNPs) associated with heightened dispositional optimism in elderly women (**Chapter 3**), while it related to fewer thoughts of hopelessness and rumination during sad mood in young women (**Chapter 4**). No association with these traits was found in the male subjects. Importantly, these traits have been identified to relate to past depression or future risk of depression and even suicide (Nolen-Hoeksema, 2000; Van der Does, 2002; Giltay et al., 2006a; Lakdawalla et al., 2007; Antypa et al., 2010a). If haplotype 2 increases MR expression in specific key brain regions, then together the data suggest that higher MR expression levels in these brain regions establishes less vulnerability for depression, particularly in women. In line with this hypothesis, several studies show that MR mRNA expression is lower in specific limbic brain structures of depressed patients (Lopez et al., 1998; Xing et al., 2004) (**Chapter 2**).

Future studies should assess whether the 5' UTR SNPs affect the MRα vs. MRβ ratio and/or the balance of the two translational isoforms MR-A vs. MR-B (van Leeuwen et al., 2010a, 2010b). Moreover, it would be important to know whether the SNPs are related to similar patterns in differential MR expression in human brain tissue. In addition, as MR expression is highly dynamic and important for coping with challenges, a thorough investigation of SNP effects on MR regulation by glucocorticoids, sex steroids or antidepressants is warranted. Important to note is that the promoter region studied here is probably not complete. For the *GR* gene multiple additional exon 1s have been identified with tissue-specific expression patterns (Turner and Muller, 2005). It is possible that the same holds true for the *MR* gene. Therefore, further studies are necessary to assess the complete structure of the *MR* promoter region and to determine whether any additional and potentially functional SNPs are linked to the SNPs described here.

To conclude, multiple high frequency SNPs along a stretch of 4 kb of the *MR* promoter region were found to constitute three common haplotypes with frequencies of .50, .41 and .09. The haplotypes were highly linked to the functional -2G/C and I180V SNPs. Haplotype 2 resulted in the highest gene transcription, which adds to the differential translational and transactivational activity due to the functional exon 2 SNPs. Possibly the promoter SNPs are to a large extent responsible for inter-individual differences in MR-related functioning found in humans, with potential consequences for stress-related pathologies.

Acknowledgements

This work was supported by the International Research Training Group (IRTG) funded by the DFG (GRH 1389/1) and NWO (DN95-420), the Netherlands Brain Foundation (Hersenstichting Nederland, 10F02(2).37), psychiatric hospital Rivierduinen and the Royal Netherlands Academy for Arts and Sciences.