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

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

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Abstract

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

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

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

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

Introduction

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

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

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

Materials and methods

Functional characterization *in vitro*

Construction of the hMR plasmids

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

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

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

Transactivation assay

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

Western blot

For western blot Cos-1 cells were seeded in 6-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 2×10^5 cells/well in DMEM supplemented with charcoal-stripped serum. The cells were transfected the next day using Trans-it Cos transfection reagent (Mirus, Madison, USA). Plasmids containing one of the hMR variants or no hMR (control) were used at 2µg/well. Cells were harvested 48 hours after transfection. The primary antibody MR 1D5 (a generous gift by Gomez-Sanchez, Division of endocrinology, University of Mississippi, Jackson, MS) was diluted 1:1000 in 0.5 % milk powder in Tris buffered saline and Tween 20 (TBST) and incubated for 1h at room temperature (RT). The secondary antibody goat anti-mouse IgG HRP was used in 1:5000 dilutions in TBST with 0.5 % milk for 1 h at RT. Tubulin was used as a control for the amount of cells and the monoclonal anti γ-Tubulin was used at a 1:1000 dilution (T6557; Sigma-Aldrich, Zwijndrecht, the Netherlands). The ECL detection system (GE healthcare, Diegem, Belgium) was used for detection. The differences in intensity of the MR bands were quantified with Image J

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

Ligand binding assay

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

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

Statistical analysis

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

Genetic association study

Recruitment

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

DNA extraction and Genotyping

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

Assessment of perceived chronic stress

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

Psychosocial stress protocol

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

Blood and Saliva Sampling

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

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

Biochemical analysis

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

Statistical analysis

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

Results

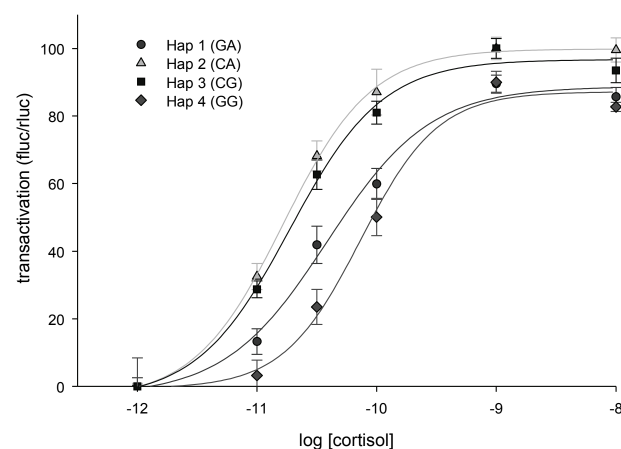
Functional characterization *in vitro*

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

Transactivation assay

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

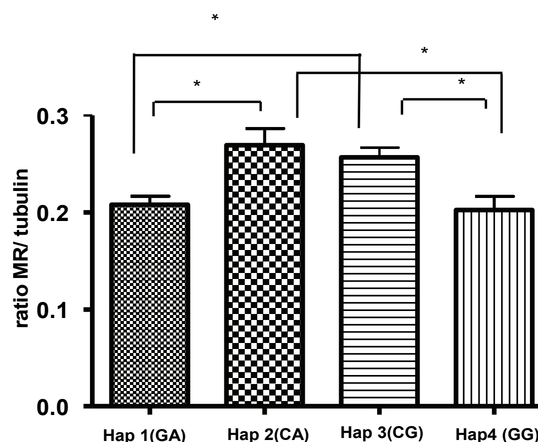
Figure 1. Cortisol induced transactivation of the four MR haplotypes on a TAT-3 promoter in Cos-1 cells. Cortisol concentrations are indicated in log units and responses are displayed as reporter (fluc)/control (rluc) ratios. The four haplotypes showed significantly different responses (p<0.0001), with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



Western blot

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

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



Ligand binding

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

Genetic association study

Genotypes and haplotypes

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

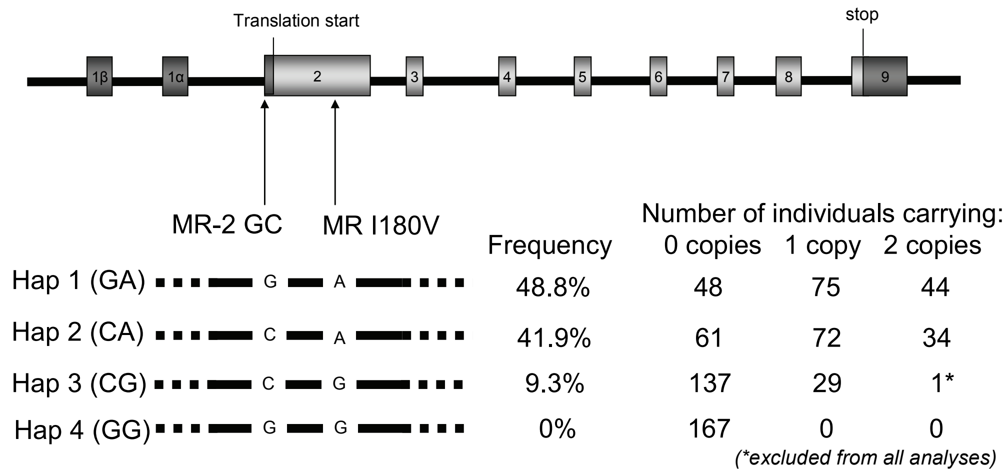


Figure 3. Schematic overview of the human MR gene (not on scale) with the location of the MR SNPs MR-2G/C and MRI80V, the haplotypes and frequencies of the haplotypes formed by these SNPs. Dark gray boxes represent untranslated exonic regions, light gray boxes represent translated exonic regions and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

Final sample

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

Perceived chronic stress

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

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

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

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

ACTH, cortisol and heart rate responses to acute psychosocial stress

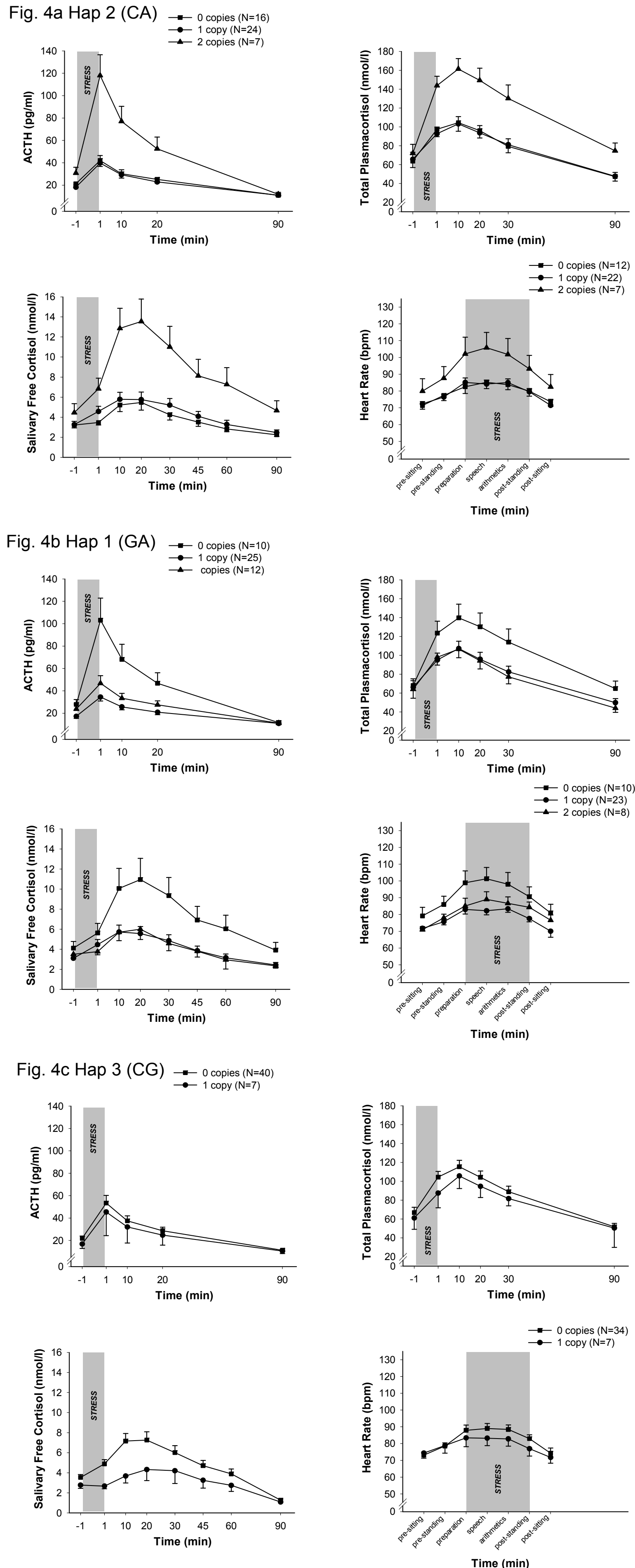
A subsample of 54 participants (20 males and 34 females) agreed to also participate in the acute stress protocol. Participants of this subsample were between 25 to 63 years of age (mean age: 48.56 ± 8.8) and had a mean body mass index (BMI) of 25.9 ± 4.8. Haplotype frequencies of this subsample were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

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

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

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

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



Discussion

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

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

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

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

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

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

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

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

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

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

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