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

Leeuwen, N. van

# 3

# Functional Mineralocorticoid Receptor (MR) gene variation influences the cortisol awakening response after dexamethasone

**Nienke van Leeuwen, Robert Kumsta, Sonja Entringer, E. Ronald de Kloet, Frans G. Zitman, Roel H. DeRijk & Stefan Wüst**

**Published in Psychoneuroendocrinology 2010**

**Summary**

Stress causes activation of the hypothalamic-pituitary-adrenal (HPA) axis and results in secretion of corticosteroids, which facilitate behavioral adaptation and promote the termination of the stress response. These actions exerted by cortisol are mediated by two brain corticosteroid receptor types: the high affinity mineralocorticoid (MR) and the lower affinity glucocorticoid receptor (GR). Dexamethasone is a potent GR agonist with affinity to MR. Administration of dexamethasone in the evening results in a significant suppression of the morning cortisol awakening response (CAR).

Here we tested the involvement of MR variants in this effect of dexamethasone in 218 young healthy subjects (125 females, all using oral contraceptives). For this purpose we determined two single nucleotide polymorphisms (SNPs) in the MR gene, the previously described MRI180V (rs5522) and the MR-2G/C (rs2070951), which both affect *in vitro* the transactivational capacity of the MR in response to either cortisol or dexamethasone.

Administration of a low dose dexamethasone (0.25mg) 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. Suppression of the CAR was highest in the female MR-2G/C GG subjects while in male GG subjects the dexamethasone suppression of the CAR was attenuated compared to the MR-2G/C GC and CC groups. For the MRI180V, male AA subjects showed after dexamethasone a higher CAR than AG subjects while this effect was not observed in females. The SNPs had no significant influence on the CAR without prior dexamethasone treatment.

The association of the CAR with functional MR gene variants only in dexamethasone treated subjects suggests the involvement of MR in dexamethasone induced suppression of morning cortisol.

**Key words**: mineralocorticoid receptor, genetic variation, HPA axis, dexamethasone, cortisol

## Introduction

Corticosteroids, secreted by the adrenals, have metabolic, immunological and cardiovascular effects and facilitate behavioural adaptation (de Kloet et al., 2005). As corticosteroid dysregulation is potentially harmful for the body, the secretion of corticosteroids as end product of the hypothalamic-pituitary-adrenal (HPA) axis is strictly regulated at several levels including the hippocampus, hypothalamus, pituitary and adrenals. Regulation of the HPA axis by cortisol is mediated in complementary fashion by two brain corticosteroid receptor types, the low affinity glucocorticoid receptor (GR, NR3C1) and the high affinity mineralocorticoid receptor (MR, NR3C2). The MR is substantially occupied under basal conditions and studies in rats suggest a role of MR in basal HPA activity (Ratka et al., 1989), particularly in feedback regulation of the pulsatile release of corticosteroids (Atkinson et al., 2008). In humans administration of a selective MR antagonist increases basal cortisol levels (Dodt et al., 1993; Born et al., 1997; Deuschle et al., 1998; Young et al., 1998; Heuser et al., 2000; Arvat et al., 2001; Otte et al., 2003a; Otte et al., 2003b; Wellhoener et al., 2004; Buckley et al., 2007). In most of these studies ACTH levels were unchanged or decreased.

The GR is mainly assumed to control feedback regulations after stress. The potent synthetic glucocorticoid dexamethasone is thought to act predominantly on the GR at the level of the pituitary gland to inhibit stress-induced HPA axis activation (De Kloet R et al., 1975). This steroid is commonly used in the dexamethasone suppression test (Carroll et al., 1968) as well as in the combined dexamethasone-CRH test. In both tests dexamethasone suppresses the HPA axis and the brain becomes depleted of the naturally occurring glucocorticoid cortisol. It is hypothesized that under those conditions the remaining low concentrations of cortisol occupy only brain MR and not GR. These tests have been frequently used to assess HPA axis regulation in psychiatric diseases. Patients with major depression do often show reduced cortisol suppression after a low dose of dexamethasone, whereas post traumatic stress disorder has been associated with an increased suppression after dexamethasone (Yehuda, 2002).

In the current study we addressed the question if the MR contributes to morning HPA axis activity and the effects of dexamethasone on this morning HPA axis activity by measuring saliva cortisol and plasma ACTH levels. There are two possibilities that may explain how the MR can influence this effect of dexamethasone. First, the residual MR occupancy after low dose dexamethasone treatment could control the drive from higher brain regions to the HPA axis (Holsboer, 2000). The second possibility is that dexamethasone, which has affinity for MR (Rupprecht et al., 1993; Grossmann et al., 2004) binds brain MR, but that this usually remains unnoticed because of the manifold higher potency of dexamethasone at the pituitary GR.

Two common functional MR gene variants, MRI180V and MR-2G/C were used to investigate the involvement of the MR on morning HPA axis activity with and without dexamethasone. Previously, we described the MR gene variant MRI180V (rs5522), a single nucleotide polymorphism (SNP) in exon 2, which results in an amino acid change in the N-terminal domain of the protein. This SNP decreases the transactivation capacity of the MR *in vitro* and was associated with enhanced cortisol and autonomic responses to an acute psychosocial stressor (DeRijk et al., 2006). In the current study

we tested a second MR gene variant called MR-2G/C, for its influence on the *in vitro* transactivation capacity with cortisol and dexamethasone as ligands. This SNP is located two nucleotides before the translation start site of the MR and might affect the translation of the MR protein.

We then tested if these two MR gene variants are associated with the cortisol awakening response (CAR) either with or without dexamethasone treatment. The CAR is a distinct rise in cortisol levels occurring in response to morning awakening (Wilhelm et al., 2007). This measure is increasingly used as an indicator of adrenocortical activity and, amongst others, the CAR was shown to be related to gender, depressive symptomatology, PTSD, primary insomnia, chronic fatigue as well as to neuroticism and perceived chronic stress (Wüst et al., 2000a; Wüst et al., 2000b; Bhagwagar et al., 2003; Pruessner et al., 2003; Backhaus et al., 2004; Roberts et al., 2004; Rohleder et al., 2004; Schlotz et al., 2004; Portella et al., 2005; Wessa et al., 2006). A moderate, yet significant heritability of the CAR was found (Wüst et al., 2000a; Wüst et al., 2000b; Bartels et al., 2003). In recent review papers a comprehensive overview of findings related to this measure and a discussion of potential mechanisms mediating the CAR can be found (Clow et al., 2004; Fries et al., 2008; Chida and Steptoe, 2009).

Thus, we investigated the association of the two MR gene variants with the CAR and early morning ACTH levels with and without dexamethasone administration.

**Materials and methods**

*Subjects*

In the present study we investigated 218 healthy subjects, 125 females and 93 males (mean age 25.1 years, standard error of mean (SEM) of ± 0.26 years; BMI 24.3 ± 0.28), who were recruited from a Trier-based community sample and from the student body of the University of Trier. All participants were non-smokers. Ethnicity was assessed by asking for the geographical origin of the subjects' parents and grandparents. Except some grandparents who originated from Poland, all subjects, parents and grandparents originated from Germany. Except for ethinyl-estradiol/progestin containing oral contraceptives (used by all females; see below for a discussion), all subjects reported to be medication free. Absence of acute or chronic diseases was confirmed in a medical examination prior to the experimental session. The protocol was approved by the ethics committee of the German Psychological Association, and written informed consent was obtained from all participants. The 218 subjects were initially selected for testing genetic variation in the GR (for details see Kumsta et al., 2007).

*Cortisol awakening response (CAR)*

Subjects were asked to collect saliva samples on two consecutive weekdays immediately after awakening and 30, 45, 60 minutes after the first sample using Salivette sampling devices (Sarstedt, Nuembrecht, Germany). They were instructed to refrain from food, drinks other than water and brushing their teeth before completion of saliva sampling.

*Dexamethasone suppression test*

Participants were instructed to ingest 0.25mg dexamethasone (Par Pharmaceutical, Spring Valley, USA) at 2300h and to collect saliva samples as described above for the assessment of the CAR after dexamethasone ingestion. Moreover, one EDTA blood sample was obtained to assess ACTH and dexamethasone levels when the subjects reported to the laboratory 60 to 90min after awakening between 0800 and 0830h.

*Biochemical analyses*

Saliva samples were stored by the subjects in their freezers and returned to the laboratory after completion of sampling. Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection as described elsewhere (Dressendorfer et al., 1992). EDTA blood samples were immediately stored on ice and centrifuged within 30min at 2000 x g and 4°C for 10min. Plasma was stored at -80°C until analysis. ACTH was measured in EDTA plasma with a chemoluminescence immunoassay (Nichols institute, Bad Nauheim, Germany). Plasma dexamethasone was assessed with an in-house RIA at the Institute of Pharmacology, University of Heidelberg. Inter-assay and intra-assay coefficients of variance were below 10% and 12%, respectively, for all analyses.

*DNA extraction and genotyping*

DNA was extracted from 10ml peripheral venous blood following a standard NaCl salting out method (Miller et al., 1988). All subjects were genotyped for the MR-2G/C and MRI180V SNPs in duplicate using 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. In addition, all the samples were genotyped with direct sequencing, therefore a PCR reaction was performed with 50ng DNA, 8.0pM of each primer (Forward: TCGCTTCTCTTGTTCT GACA, Reverse: CTGGACTCATGCTTCCTTGT) and AmpliTaq Gold (Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands), according to the manufacturer's protocol. Sequencing of the PCR product was done with BigDye Terminator Sequencing Kit v3.1(Applied Biosystems Nieuwekerk a/d IJssel, The Netherlands) on an ABI Prism 3100-Avant Genetic Analyzer, according to the manufacturer's instructions, while sequences were analyzed using Vector NTI (Invitrogen).

*Site directed mutagenesis of hMR*

The recombinant pRSV human MR plasmid, containing the last 30 base pairs of exon 1α into the nontranslated region of exon 9, was obtained from Dr. R. Evans (gene expression laboratory and HHMI, The Salk Institute for Biological Studies, La Jolla, Ca). The rs2070951 site was mutated from G to C with the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) using the primers 5'-GGCCGAGGCAGCGATGGAGACCAAAG-3' and 5'-CGCTGCCTCGGCCCTTTGGTCTCCAT-3' according to the manufacturers protocol. After mutagenesis the hMR insert of the plasmid was sequenced to assure absence of other mutations. Plasmids were purified from DH5α E-coli bacterial cultures using the Pure Yield purification system (Promega, Leiden, The Netherlands).

*Transfections and luciferase assays*

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). Plasmids containing one of the hMR variants or no hMR (control) and the reporter plasmid 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) for normalization purposes. One day after transfection, the cells were treated with either cortisol (Sigma-Aldrich, Zwijndrecht, the Netherlands) or dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands) in the concentrations 0, $10^{-11}$, $3.10^{-11}$, $10^{-10}$, $3.10^{-10}$, $10^{-9}$ and $10^{-8}$ M. After 24h of incubation the cells were harvested in passive lyses buffer (Promega) and firefly and Renilla luciferase activity was determined using the dual label reporter assay (Promega) and a luminometer (CENTRO XS3 LB960, Berthold, Bad Wildbad, Germany).

*Statistical analyses*

In the *in vitro* assays the firefly/renilla luciferase ratios were normalized to both the lowest and highest luciferase ratio for each hMR variant, background expression was subtracted and differences between the hMR variants were analyzed with a two-way ANOVA with Bonferroni posttest and by comparing the EC50 and slope of the dose response curves with a t-test using GraphPad prism 4 (GraphPad software Inc, San Diego, CA). Haploview (Barrett et al., 2005) was used to calculate Hardy Weinberg equilibrium (HWE) and linkage disequilibrium among the two MR SNPs (estimated with D' and $r^2$). To test if the MR genotypes are equally distributed in the different GR genotype groups a $X^2$ test was computed. Cortisol data were log transformed to yield unskewed outcome variables. The two CAR measurements (without prior dexamethasone administration) were averaged in order to enhance the reliability of the measure (Wüst et al., 2000b; Hellhammer et al., 2007). General linear models (GLMs) were computed to assess the repeated measures effect *time*, the between-subjects effect *genotype* as well as the interaction *time x genotype* for the CAR with and without dexamethasone pretreatment. In order to reveal *sex x genotype* interactions, sex was also included as predictor in the GLMs. Greenhouse-Geisser corrections were applied where appropriate, and only adjusted results are reported. One-way ANOVAs were performed to compare mean plasma dexamethasone and ACTH levels after dexamethasone administration between comparison groups. All results shown are the mean ± SEM unless otherwise stated. Apart from the exceptions stated above statistical analyses were performed using SPSS 14.0 (Chicago, IL, USA). While statistical analyses of cortisol levels were based on log-transformed data, figures show untransformed means in order to provide a more naturalistic impression of cortisol levels.

**Results**

*In vitro functionality testing*

Cos-1 cells were transfected with plasmids containing either no MR, the MR-2C or MR-2G nucleotide. Each plasmid was three times cultured, purified and tested to exclude variation due to

impurity or concentration of the plasmid. In each of the three experiments the plasmids were tested in triplicate. After incubation with either cortisol or dexamethasone at 7 concentrations in the range from 0 to $10^{-8}$M luciferase activity was measured. The negative control, cells transfected with plasmids containing no MR, showed only with the highest ($10^{-8}$M) dexamethasone concentration an induction of 12%. For the other dexamethasone concentrations and all the cortisol concentrations there was no induction of luciferase in the negative control. After normalization of the luciferase data the 12% background induction was subtracted.

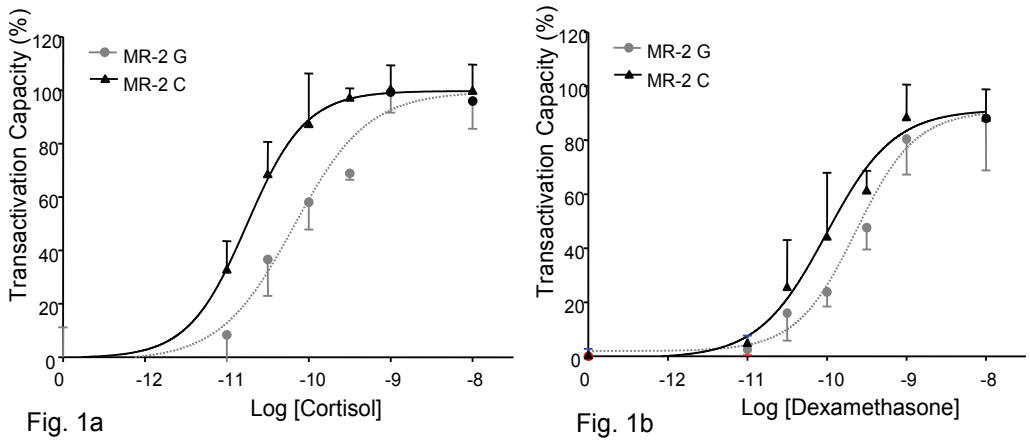


**Figure 1** a) Cortisol and b) dexamethasone driven transactivation capacity of the MR -2G/C variants on a TAT-3 promoter in Cos-1 cells displayed as percentage of the maximal transactivation capacity (±SD) – the background activity when no MR is present. Cortisol and dexamethasone concentrations are indicated in log units. The MR-2G variant (dashed circle) showed a lower transactivation capacity compared with the MR-2C variant (black triangle) (cortisol $p<0.001$; dexamethasone $p<0.01$).

The MR-2C variant showed significantly higher transcriptional activity than the MR-2G variant using either cortisol or dexamethasone ($F_{1,86}=52.3$, $p<0.001$ for cortisol and $F_{1,86}=7.3$, $p=0.01$ for dexamethasone, Figure 1a & b). Post hoc analysis showed significant genotype effects at cortisol concentrations of $3.10^{-11}$, $10^{-11}$, $10^{-10}$ and $3.10^{-10}$M ($t=4.265$, $t=5.770$, $t=5.297$, all $p<0.001$ and $t=3.11$ with $p<0.05$, respectively) and at a dexamethasone concentration of $10^{-10}$ ($t=3.26$ and $p<0.05$). Analysis of the dose response curves showed a significant difference in the EC50 between the two MR variants (cortisol $t_{14}=5.816$, $p<0.0001$ and dexamethasone $t_{14}=2.747$, $p=0.016$) but no difference in the slope of the curves. For cortisol the EC50 was $6.6.10^{-11}$ for MR-2 G and $1.7.10^{-11}$ for MR-2 C and for dexamethasone the EC50s were $2.3.10^{-10}$ and $9.9.10^{-11}$ respectively.

*Genotyping*

The 218 subjects were genotyped for the polymorphisms MRI180V and MR-2G/C with a Taqman assay and by direct sequencing yielding identical genotype frequencies (Table 1). Observed allele frequencies corresponded to those previously reported (DeRijk et al., 2006; Kuningas et al., 2007). While the MRI180V SNP was in Hardy Weinberg equilibrium (HWE) a deviation from HWE was detected for the MR-2G/C ($p=0.0153$; see below for a discussion). Estimated linkage between MRI180V and MR-2G/C was D'=1 (conf bounds 0.76-1) and $r^2=0.114$. Although the genes for the GR and the MR are located on different chromosomes (5q31.3 vs 4q31.1) a $X^2$ test was computed

confirming that the investigated MR SNPs were not statistically related to the GR SNPs that were determined in the same cohort ($X^2_{(20)}$=19.72, p=0.475; see Kumsta et al., 2007).

*Table 1*
Observed genotypes

| | MRI180V | | ∑ |
|---|---|---|---|
| | **AA** | **AG** | |
| **MR-2 CC** | | | |
| Male | 12 | 6 | 18 |
| Female | 17 | 13 | 30 |
| Total | 29 (13.3%) | 19 (8.7%) | 48 (22.0%) |
| **MR-2 CG** | | | |
| Male | 43 | 11 | 54 |
| Female | 59 | 15 | 74 |
| Total | 102 (46.8%) | 26 (11.9%) | 128 (58.7%) |
| **MR-2 GG** | | | |
| Male | 21 | 0 | 21 |
| Female | 21 | 0 | 21 |
| Total | 42 (19.3%) | 0 (0%) | 42 (19.3%) |
| ∑ | 173 (79.4%) | 45 (20.6%) | **218 (100%)** |

*Cortisol awakening response (CAR) with and without prior dexamethasone administration*
As previously described the CAR differed significantly between males and females, with males showing higher responses. Therefore sex was included as a predictor in all further analyses. The CAR without prior dexamethasone treatment showed the expected mean rise. However, we did not find any significant association between MR genotypes or haplotypes and the cortisol increase after awakening. The only noteworthy statistical trends were the interaction *genotype x sex* ($F_{2,204}$=1.62, p=0.20) for the MR-2G/C (Fig. 2b) and the interaction *time x genotype x sex* ($F_{1.98,\ 408.6}$=2.38, p=0.09) for MRI180V (Fig. 2a), suggesting a somewhat higher CAR in male MRI180V AA subjects (see also Table 2 for an overview of effects).

The assessment of plasma dexamethasone levels confirmed that all participants had ingested the dexamethasone tablet and no significant difference between males and females was observed (males: 168 ± 51, females 174 ± 40 ng/100 ml, $F_{2,212}$=.863, p=0.4). Moreover, individual dexamethasone levels were not associated with the MR genotypes (all F<1.05 and all p>.39). After 0.25mg dexamethasone the CAR was still observed but cortisol concentrations were significantly lower then without prior dexamethasone treatment (main effect *treatment:* $F_{1,189}$=597.8, p<.001). The average starting level was reduced from 11.2 ± 5.1 to 2.9 ± 1.8 nmol/l (74% suppression) and the average peak level from 17.0 ± 6.9 to 4.5 ± 2.3 nmol/l (74% suppression). Furthermore, cortisol levels in females (all using OCs) were significantly lower than in males (main effect *sex:* $F_{1,196}$=36.69, p<.001)

Both MR SNPs were associated with post-dexamethasone cortisol levels, measured as the CAR, in a sex specific way (see Fig. 3a & b). In males MRI180V AA subjects (homozygotes major allele) showed a clearly higher (i.e. less suppressed) mean CAR than MRI180V G carriers, while this effect could not be observed in females resulting in a significant *genotype x sex* interaction ($F_{1,193}$=4.34, p=0.039). Also independent of the subjects' gender we found a trend towards a main effect *genotype* ($F_{1,193}$=2.84, p=0.093) but this trend was obviously driven by the large difference in males. Accordingly, this main effect *genotype* was significant when females were excluded in a supplementary analysis ($F_{1,80}$=5.06, p=0.027). For the MR-2G/C an even clearer sex specific effect was detected with GG subjects showing the largest CAR in males and the most suppressed CAR in females (interaction *genotype x sex*: $F_{2,191}$=4.23, p=0.016).

As MRI180V and MR-2G/C are in linkage disequilibrium an additional analysis based on haplotypes was performed (see Fig. 3c). Three haplotypes were detected, MR-2-G/I180V-A, MR-2-C/I180V-A and MR-2-C/I180V-G and the results of the haplotype analysis confirmed the genotype based comparisons. A significant *genotype x sex* interaction was detected *(*$F_{4,187}$=2.84, p=0.026) and again this interaction was primarily based on the difference between male and female MR-2G/C GG subjects. A remarkable comparison group in this analysis were the male MR-2CC/I180V-AG subjects who showed a virtually completely suppressed CAR (see also Table 2 for an overview of effects).

**Table 2.** General linear model effects for the CAR with and without dexamethasone pre-treatment

| | | CAR | | | CAR DEX | | |
|---|---|---|---|---|---|---|---|
| | **Effect** | **df** | **F** | **p** | **df** | **F** | **p** |
| MR-2G/C | Genotype | 2;204 | 1.53 | .220 | 2;191 | 0.26 | .974 |
| | Sex | 1;204 | 2.14 | .145 | 1;191 | 37.18 | <.001 |
| | Genotype x Sex | 2;204 | 1.62 | .200 | 2;191 | 4.23 | .016 |
| | Time | 1.96;400.73 | 77.57 | <.001 | 2.15;410.01 | 66.89 | <.001 |
| | Time x Genotype | 3.93;400.73 | 0.70 | .593 | 4.29;410.01 | 1.36 | .244 |
| | Time x Sex | 1.96;400.73 | 16.90 | <.001 | 2.15;410.01 | 10.07 | <.001 |
| | Time x Genotype x Sex | 3.93;400.73 | 0.68 | .601 | 4.29;410.01 | 1.57 | .179 |
| MRI180V | Genotype | 1;206 | 0.56 | .457 | 1;193 | 2.84 | .093 |
| | Sex | 1;206 | 0.31 | .575 | 1;193 | 10.94 | .001 |
| | Genotype x Sex | 1;206 | 0.67 | .414 | 1;193 | 4.34 | .039 |
| | Time | 1.98;408.56 | 71.13 | <.001 | 2.16;416.02 | 49.14 | <.001 |
| | Time x Genotype | 1.98;408.56 | 0.49 | .610 | 2.16;416.02 | 1.83 | .158 |
| | Time x Sex | 1.98;408.56 | 8.61 | <.001 | 2.16;416.02 | 3.63 | .024 |
| | Time x Genotype x Sex | 1.98;408.56 | 2.38 | .094 | 2.16;416.02 | 0.98 | .38 |
| Haplotypes | Genotype | 4;200 | 1.30 | .270 | 4;187 | 1.02 | .40 |
| | Sex | 1;200 | 0.66 | .416 | 1;187 | 15.01 | <.001 |
| | Genotype x Sex | 4;200 | 0.93 | .448 | 4;187 | 2.84 | .026 |
| | Time | 1.98;395.98 | 69.03 | <.001 | 2.14;399.94 | 48.51 | <.001 |
| | Time x Genotype | 7.92;395.98 | 0.77 | .625 | 8.56;399.94 | 1.60 | .117 |
| | Time x Sex | 1.98;395.98 | 9.55 | <.001 | 2.14;399.94 | 3.82 | .020 |
| | Time x Genotype x Sex | 7.92;395.98 | 1.13 | .340 | 8.55;399.94 | 1.29 | .244 |

ACTH levels, assessed between 0800h and 0830h after dexamethasone administration were significantly more suppressed in females (all using OCs) than in males (main effect *sex:* $F_{1,213}$=21.56, p<.001). We did, however, not detect any significant association between ACTH levels and MR genotype (MRI180V: means±SEM: 31.18±1.80 for AA, 35.35±5.33 for AG; main effect *genotype:* $F_{1,212}$=0.95, p=0.33; interaction *genotype x sex*: $F_{1,212}$=1.28, p=0.26; MR-2G/C: means±SEM: 28.68±4.23 for CC, 33.06±2.94 for GC, 32.68±5.17 for GG; main effect *genotype:* $F_{2,212}$=0.17, p=0.84; interaction *genotype x sex*: $F_{2,212}$=0.56, p=0.95; haplotypes: means±SEM: 32.02±6.05 for CC/AA, 30.36±3.02 for GC/AA, 32.68±5.17 for GG/AA, 23.49±5.54 for CC/AG, 43.57±8.54 for GC/AG; main effect *genotype:* $F_{4,212}$=1.29, p=0.27; interaction *genotype x sex*: $F_{4,212}$=0.24, p=0.92).

**Figure 2** (next page) Mean cortisol awakening responses (± SEM) in males and females with the MR genotype **(a)** I180V AA (males n= 76, females n= 97) and I180V AG (males n= 17, females n= 28); **(b)** -2CC (males n= 18, females n= 30), -2GC (males n= 54, females n= 74) and -2GG (males n= 21, females n= 21); **(c)** MR haplotypes -2CC/ I180V AA (males n= 12, females n=17), -2GC/ I180V AA (males n= 43, females n= 59), -2GG/ I180V AA (males n= 21, females n=21), -2CC/ I180V AG (males n= 6, females n= 13) and -2GC/ I180V AG (males n= 11, females n= 15).

**Figure 3** (next page) Mean cortisol awakening responses (± SEM) after 0.25mg dexamethasone at 2300h the evening before in males and females with the MR genotype **(a)** I180V AA (males n= 76, females n= 97) and I180V AG (males n= 17, females n= 28) ; **(b)** -2CC (males n= 18, females n= 30), -2GC (males n= 54, females n= 74) and -2GG (males n= 21, females n= 21); **(c)** MR haplotypes -2CC/ I180V AA (males n= 12, females n=17), -2GC/ I180V AA (males n= 43, females n= 59), -2GG/ I180V AA/ (males n= 21, females n=21), -2CC/ I180V AG (males n= 6, females n= 13) and -2GC/ I180V AG (males n= 11, females n= 15). Please note the different y-axes scales in Fig. 2 and 3.

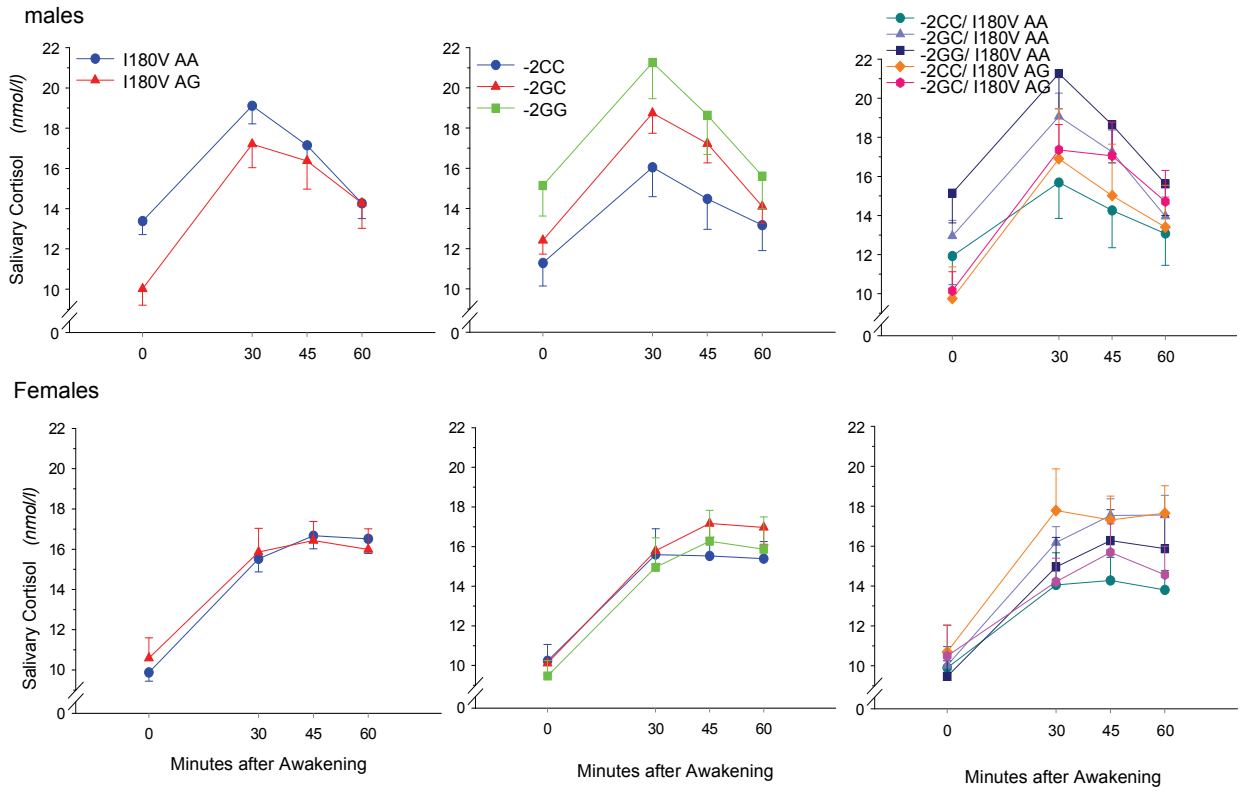
males
Salivary Cortisol (nmol/l)
I180V AA
I180V AG
-2CC
-2GC
-2GG
-2CC/ I180V AA
-2GC/ I180V AA
-2GG/ I180V AA
-2CC/ I180V AG
-2GC/ I180V AG
Females
Minutes after Awakening

**Fig. 2**

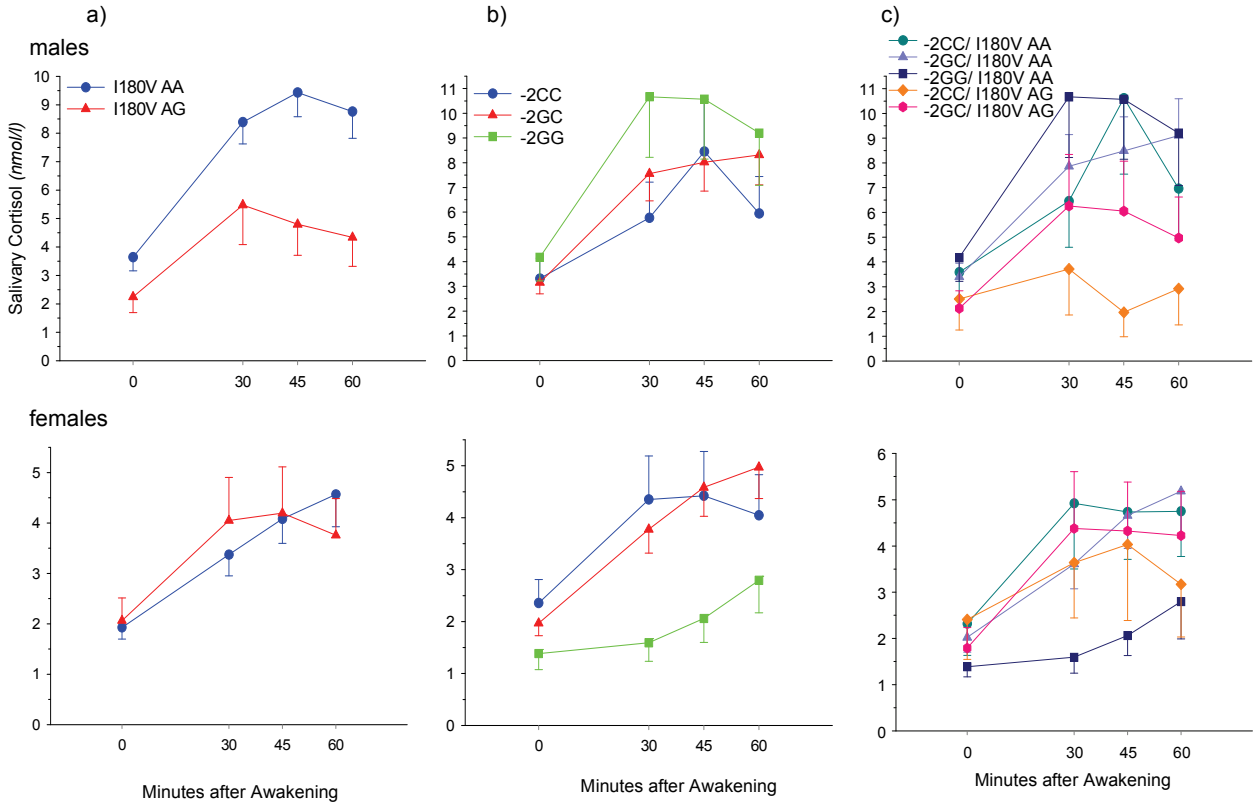
a)
b)
c)
males
females
Salivary Cortisol (nmol/l)
I180V AA
I180V AG
-2CC
-2GC
-2GG
-2CC/ I180V AA
-2GC/ I180V AA
-2GG/ I180V AA
-2CC/ I180V AG
-2GC/ I180V AG
Minutes after Awakening

**Fig 3.**

**Discussion**

Two *in vitro* functional MR gene variants, MRI180V and MR-2G/C were associated with suppression of the cortisol awakening response following administration of 0.25mg dexamethasone. This effect was sex specific and was also observed with the haplotype of both SNPs. Moreover, when males were analyzed separately, a main effect for the MRI180V was observed. However, neither the two SNPs nor the haplotype were significantly related to awakening cortisol responses without prior dexamethasone treatment.

Previously, we showed that the minor allele of the MR180V results in a relative loss of function *in vitro* using cortisol as ligand and although we did not show the data, we observed this as well with our assay. The present study demonstrates that the MR-2C variant has a significantly higher capacity in the activation of TAT-3 regulated gene transcription, as compared to the MR-2G, using either cortisol or dexamethasone as ligands. MR-2G/C is located two nucleotides before the first translation startsite in the kozak region, which is important for translation of the MR. The MR contains two translation start sites resulting in the isoforms MR-A and MR-B (Pascual-Le Tallec et al., 2004). MR-A is more potent in activating a MMTV or a GRE containing promoter than MR-B when aldosterone is used as a ligand. The wild type plasmid, expressing both MR-A and MR-B, has an even higher transactivation capacity than MR-A. Therefore, it can be hypothesized that the ratio MR-A/ MR-B might be important. This MR-2G/C SNP might change this MR-A / MR-B ratio and thereby the transactivation capacity. The observed effects of the studied SNPs *in vitro* indicate that they can be functional *in vivo*, although the sex specific effects observed in the association study indicate that a precise prediction of *in vivo* consequences from *in vitro* data is very difficult. On the other hand, these functional genetic variants provide natural tools to study the involvement of the MR in HPA axis regulation.

Dexamethasone activates GR exerting long lasting genomic effects on negative feedback. This occurs predominantly at the pituitary level and it causes suppression of HPA axis activity until the next morning. However, the differential effects of functional MR gene variants on dexamethasone suppression of cortisol levels indicate the involvement of the MR. Several mechanisms can account for this observation.

First, the MR gene variants, as *in vitro*, are expected to react differently *in vivo* to stimulation with dexamethasone and thus also differentially affect HPA axis suppression. This is a likely explanation, since without dexamethasone there was no genotype effect on the CAR. Moreover, dexamethasone has an appreciable affinity fir the MR (Rupprecht et al., 1993; Grossmann et al., 2004). Although likely, a mechanism assuming direct MR binding of dexamethasone needs further investigation. Central brain MR are less 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 might pass the blood brain barrier and activate MR, while the pituitary gland also contains some MR.

Second, 0.25mg dexamethasone results in lower, but still appreciable levels of saliva cortisol (Fig. 3). The levels are comparable with the levels normally observed in the afternoon and are probably sufficient to activate MR (Meijer et al., 1998; Karssen et al., 2005; Droste et al., 2008). 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 (Bradbury et al., 1994; Tajima et al., 1999; Kovacs et al., 2000). Since limbic MR has inhibitory effects on the HPA axis, a reduced MR activation may result in this enhanced drive (Holsboer, 2000). However, we did not observe a significant association with ACTH levels collected between 0800h and 0830h, the reason for this inconsistency is not clear. As the subjects awoke at least one hour prior to blood sampling the ACTH measures reflect hormonal levels after the CAR. It could thus be speculated that an association with the MR genotype can be detected when the awakening response (cortisol or ACTH) is assessed but not when single early morning levels are considered.

Finally, fast non-genomic actions of membrane bound MR have been described. This membrane bound MR has much lower affinity for corticosterone then the classic MR with its genomic actions (Karst and Joels, 2005; Joels et al., 2008) 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.

Changes in cortisol suppression after dexamethasone have been associated with psychopathology such as PTSD and major depression. The involvement of MR variants in the dexamethasone induced suppression of the CAR is an indication that those MR variants might be involved in psychopathology as well. A previous study by Kuningas et al supports this hypothesis since they found an association between MRI180V and feelings of depression in a cohort consisting of elderly (Kuningas et al., 2007). However, we used the CAR as a more precise measure of dexamethasone induced suppression of cortisol instead of one cortisol sample taken in the morning. The functionality of the CAR (with or without dexamethasone administration) for pathology is not completely understood yet, making it difficult to draw more far-reaching conclusions.

Sex differences in HPA axis basal activity, regulation and reactivity are well known (Kudielka and Kirschbaum, 2005). Our previous study using the same cohort revealed a sex specific association between the GR *Bcl*I SNP and cortisol responses to a psychosocial stressor (Kumsta et al., 2007). Moreover, the GR SNP 9βAG was significantly associated with morning ACTH levels after dexamethasone administration, but only in males. In the current study, the association with post dexamethasone cortisol concentrations in males seems to be due to the MRI180V SNP. In contrast, the MR-2G/C SNP had the largest effect in females with the MR-2GG group showing a suppressed CAR. Thus it seems that the different variants of the MR gene can have opposite effects in males and females.

Since the MR has high affinity for aldosterone, cortisol, deoxycortisol and progesterone (Rupprecht et al., 1993), female hormones may interfere with actions mediated by cortisol. For example, expression of the MR is influenced by estrogens, while progesterone affects the binding of corticosterone in rat hippocampus (Carey et al., 1995). In rats, estrogens decrease the expression of the MR in the pituitary (Turner, 1990) and progesterone treatment increased the activity of the MR promoter in cell lines (Castren et al., 1995). Interestingly, after adrenalectomy in rats testosterone

rather then corticosterone regulates AVP expression (Viau et al., 1999). Furthermore, we recently showed that MRI180V interacts differentially with different ligands. While the MRI180V modulates cortisol dependent transactivation *in vitro*, no difference was observed with aldosterone as a ligand (DeRijk et al., 2006). Thus both unique ligand – MR gene variant interactions and sex hormone modulation of MR gene expression could underlie the gender specific interactions of MR gene variants on post-dexamethasone cortisol levels.

It is a certain limitation of our study that we could not control for the subjects' compliance with the saliva sampling protocol. In studies that used collection devices equipped with electronic sensors it was revealed that strict compliance with instructions cannot be granted in ambulatory settings (Kudielka et al., 2003; Broderick et al., 2004). However, such an inaccuracy usually affects only a relatively small proportion of measurements, on average the ambulatory CAR is identical with the CAR assessed under sleep laboratory conditions (Wilhelm et al., 2007) and – most importantly - it is extremely unlikely that possible sampling time inaccuracies are systematically associated with our MR genotype groups.

Another limitation of our study concerns the interpretability of the observed sex by genotype effects since all female participants used oral contraceptives (OCs). We chose to investigate only oral contraceptive using women as the complete study protocol included the exposure to a laboratory stress protocol (for details see (Kumsta et al., 2007) and in order to avoid a modulation of cortisol and ACTH responses to acute stress by the menstrual cycle phase (Kirschbaum et al., 1999).

It is well known that ethinyl-estradiol leads to increased corticosteroid binding globulin (CBG) synthesis, which in turn influences total serum cortisol levels and the availability of free cortisol. Indeed, CBG was shown to modulate hormonal responses to TSST exposure in oral contraceptive using females, as well as in males (Kumsta et al., 2007). However, the CAR (without dexamethasone pretreatment) was found to be not significantly affected by OCs in a relatively large cohort of 509 subjects (319 females, 29% used OCs) (Wüst et al., 2000b).

Data on a potential influence of OCs on HPA axis hormones after dexamethasone administration are surprisingly scarce and we cannot rule out such an effect in our study. However, after 1mg dexamethasone at 2300h free cortisol levels at 1600h did not significantly differ in women taking contraceptives compared to women in various stages of the menstrual cycle (Ansseau et al., 1993). Furthermore, at least the absolute levels of CBG per se are likely not responsible for our observed genotype by sex interactions, since we did not observe different CBG levels in the MR genotype groups (data not shown).

It could be argued that different OC brands differing in compositions could have differential effects on our measures. However, it appears rather unlikely that the usage of different OC brands was a major confounder in the present study. Females in our sample used 14 different OC brands (all monophasic) with 11 different compositions and we did not even detect a weak trend for a significant association between OC composition and the CAR, the CAR after dexamethasone or ACTH levels after dexamethasone administration. All main effects and interactions had a p value >.38. As most OC preparations contain .03mg ethinyl-estradiol we performed a further

supplementary analysis and compared females using OCs with .03mg, with less than .03mg and with more than 0.03mg ethinyl-estradiol. Again, we did not detect a significant effect on our measures (lowest p value: .25).

Generalization of our findings on women not taking oral contraceptives is not possible and this is a clear weakness of our study, which was accepted on the basis of feasibility considerations when the study was designed. It should be noted though, that more than 60% of young females (16-30 yrs) in Germany use OCs (von Have et al., 2005) and similar frequencies can be found in other industrialized countries. We thus think that studying OC females is a relevant topic.

The two investigated SNPs in the MR, MRI180V and MR-2G/C showed linkage disequilibrium (D'=1 (conf bounds 0.76-1) and $r^2$=0.114). This observed linkage has implications for the analysis because a combination of SNPs, a so called haplotype, can respond differently compared to isolated SNPs. Again a sex by genotype interaction was found for the association with post dexamethasone cortisol levels. Females with the MR-2/I180V GG/AA haplotype showed the lowest CAR, with no significant rise in cortisol at all, while men with this haplotype had the highest CAR. No unexpected interaction of the two SNPs, MRI180V & MR-2G/C, making the haplotypes was observed; both male MRI180V AG and MR-2G/C CC carriers showed lowest cortisol levels as well as the haplotype MRI180V AG/MR-2CC. In females the MR-2G/C made the difference, in contrast to the MRI180V, with its effect coming back in the haplotype, with the MR-2GG showing lowest cortisol levels. However, due to the rather small size of the haplotype groups these observations should be interpreted with caution. Subjects with MR-2 GG/MRI180V AG or MR-2 GG/MRI180V GG genotype combinations were not observed in this population and as part of a different study we sequenced several hundreds additional subjects and again these genotype combinations were not found (unpublished). This is reflected in the LD that was observed between the two SNPs.

The MR-2G/C SNP deviated from Hardy Weinberg equilibrium in the current cohort. This is often a result of a genotyping error but in this study we used two different methods for genotyping and found identical results, excluding a technical error. Furthermore, all individuals reported to be from central European descent making population stratification unlikely. The remaining explanations are random sample variation or the non-random selection of the cohort. The cohort was selected based on GR polymorphisms. The genes for the GR and the MR are located on different chromosomes (5q31.3 vs 4q31.1) therefore it is not expected that this selection influences the MR genotype distribution and indeed a $X^2$ test confirmed that the investigated MR SNPs were not statistically related to the GR SNPs. We only compared the genotype and haplotype effects on cortisol within this group and we did not perform a case-control study with a comparison between groups. In addition, the genotypes and haplotypes were used in the analysis instead of alleles. Therefore it is unlikely that this deviation from HWE had a major confounding impact on the findings. (Minelli et al., 2008).

In summary, the data suggest a role of the MR in the control of negative feedback of the HPA axis as tested with dexamethasone, but how the MR variants precisely modulate this feedback action remains to be established. The observed effects of these common functional variants of the human MR gene are gender specific.