Mineralocorticoid receptor in human brain : a key player in resilience

Klok, M.D.

Part 3

Genetic association studies among depressed patients

Common and functional *MR* gene variants are know to associate with variability in HPA axis activity under non-stress and stressful conditions in healthy individuals. Here we tested for association with HPA axis activity in depressed subjects (**Chapter 6**).

We have found that the common and functional *MR* gene variants modulate neuroendocrine control, personality and cognitive reactivity. As a final study these *MR* gene variants were tested for association with the risk of depression (**Chapter 7**).

Chapter 6

Common functional mineralocorticoid receptor polymorphisms modulate the cortisol awakening response: interaction with SSRIs

Melanie D. Klok, Sophie A. Vreeburg, Brenda W.J.H. Penninx, Frans G. Zitman, E. Ron de Kloet, Roel H. DeRijk

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Abstract

Background: Cortisol controls the activity of the hypothalamic-pituitary-adrenal (HPA) axis during stress and during the circadian cycle through central mineralocorticoid (MR) and glucocorticoid receptors (GR). Changes in MR and GR functioning, therefore, may affect HPA axis activity. In this study we examined the effect of common functional *MR* gene variants on the cortisol awakening response (CAR), which is often disturbed in stress-related disorders like depression.
Methods: Common functional *MR* single nucleotide polymorphisms (SNPs; *MR* -2G/C and I180V) and haplotypes were tested for association with variability in the CAR in a large cohort (Netherlands Study of Depression and Anxiety, NESDA) of patients diagnosed with a lifetime major depressive disorder (MDD). Saliva cortisol measurements and genotypes could be obtained from a total of 1026 individuals, including 324 males and 702 females.
Results: The *MR* -2C/C genotype was associated with an attenuated CAR increase in women ($p = .03$) but not in men ($p = .18$; $p = .01$ for SNP-by-sex interaction). The *MR* I180V SNP had no significant effect on the CAR. Additional analysis revealed that effect of the -2G/C SNP on the CAR was due to an interaction with frequent use of selective serotonin reuptake inhibitors (SSRIs). Only in subjects using SSRIs (men and women) highest total morning cortisol levels were observed in -2G/G carriers, while the CAR was completely flattened in women with the -2C/C genotype ($p < .05$). The results were independent of multiple potential confounders and had an effect size of $r = .14$ — $.27$.
Conclusions: This study shows that the *MR* -2G/C SNP modulated the CAR only in the MDD patients using SSRIs, with a clear allele-dose effect in women. This suggests that effect of SSRIs on cortisol regulation depends in part on the *MR* genotype with possible implications for future treatment selection.

Keywords: mineralocorticoid receptor, single nucleotide polymorphism (SNP), cortisol awakening response (CAR), interaction

Introduction

Optimal regulation of cortisol levels by the hypothalamic-pituitary-adrenal (HPA) axis is crucial for adaptive physical and psychological responsiveness to everyday challenges (De Kloet et al., 1998). Hence, disturbances in activity of the HPA axis may develop into various disorders, including major depressive disorder (MDD) (Nestler et al., 2002), while normalization of HPA axis parameters preceding clinical relief is often observed (Barden et al., 1995; Zobel et al., 2004). These changes in HPA axis activity depend on the feedback action of cortisol, which is mediated by two brain corticosteroid receptors, i.e. the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR).

Due to its low affinity the GR only becomes activated when cortisol levels are high, as occurs during stress and at the peaks of the ultradian rhythm during the circadian cycle (de Kloet and Sarabdjitsingh, 2008). Through the GR stress-induced cortisol levels are suppressed. The MR has a high affinity for cortisol and therefore remains already highly occupied throughout the day under non-stress, basal conditions. During the day the MR exerts a tonic inhibition on circulating cortisol levels (de Kloet et al., 1998). Administration of a MR antagonist to both animals and humans increases diurnal plasma corticosteroid levels by enhancing the amplitude of the corticosteroid pulses (Heuser et al., 2000; Atkinson et al., 2008). In addition, the MR potentiates the initial neuroendocrine stress reaction. In response to stress the MR and GR mediate in complementary fashion the action of cortisol from the initial stress reaction to the management of later adaptive phases. Recently, besides a cytoplasmic high affinity MR also a low affinity membrane MR was identified, however, the specific roles of these distinctly localized receptors in HPA axis activity still has to be assessed (Joels et al., 2008). What the specific roles of the MR and GR are in regulating the circadian peak is also still unclear. Because of its high affinity it is likely that the MR is implicated. Moreover, the question remains whether cortisol levels at the circadian peak are high enough to actually bind to the GR. In the present study the focus is on the MR.

By examining the effect of common functional *MR* gene variants, we showed that *MR* genetic variability confers inter-individual differences in neuroendocrine regulation under both basal non-stress conditions and after stress (DeRijk et al., 2006; Kuningas et al., 2007; van Leeuwen et al., 2010a, 2011). For the *MR* gene, two functional SNPs (*MR* I180V and -2G/C) have been described so far, both affecting MR expression and/or gene transactivation in cell lines. The V-allele of the *MR* I180V SNP results in a higher cortisol response to the Trier Social Stress Test (TSST) (DeRijk et al., 2006), which was accompanied by an increased heart rate response. In a different study, the C-allele of the *MR* -2G/C SNP was found to be associated with lower plasma cortisol levels in the morning among healthy elderly (Kuningas et al., 2007). Together, these data indicate that both basal non-stress and stress-induced HPA regulation may vary in part due to differences in MR activity. As yet, it is still unclear to what extent the MR (and GR) influences the cortisol awakening response (CAR). In a recent study, both known *MR* SNPs were found to affect the CAR in healthy individuals, although effects were only significant after dexamethasone treatment and were sex dependent (van Leeuwen et al., 2010a).

The CAR consists of a distinct rise in cortisol levels directly after awakening, which reaches peak levels at 30 min and returns to baseline levels 60 min after awakening (Pruessner et al., 1997; Wust et al., 2000b; Wilhelm et al., 2007). The CAR is considered as a response to awakening, superimposed on the ultradian rhythm during the circadian cycle (Kuehner et al., 2007). Because of its intra-individual stability, the CAR is thought of as a trait measure for HPA axis activity (Pruessner et al., 1997; Wust et al., 2000a) and appears to be influenced in part by genetic factors (Wust et al., 2000a). Sociodemographic, lifestyle and sleep factors, chronic stress and daily hassles all may modulate the CAR (Pruessner et al., 1997; Wust et al., 2000a, 2000b; Buchanan et al., 2004; Hellhammer et al., 2007; Fries et al., 2009; Vreeburg et al., 2009b).

Major depressive disorder (MDD) is in many cases associated with hyperactivity of the HPA axis (Nestler et al., 2002), including an enhanced CAR in both remitted and current depressed patients (Vreeburg et al., 2009a). Normalization of HPA axis reactivity often occurs after treatment with antidepressants (Barden et al., 1995; Zobel et al., 2004), while antidepressants themselves were found in animal studies and cell lines to increase the expression of the MR and/or GR (Seckl and Fink, 1992; Holsboer and Barden, 1996; Bjartmar et al., 2000). Moreover, MR antagonists diminish, while MR agonists enhance the efficacy of a tricyclic antidepressant (TCA) or a selective serotonin reuptake inhibitor (SSRI) respectively (Holsboer, 1999; Otte et al., 2010). Collectively, these data imply an important role for the efficiency of MR signaling in changing HPA axis activity, pathogenesis and with consequences for treatment.

Here we tested the hypothesis that genetic variants of the *MR* gene relate to variability in the CAR in lifetime MDD patients. To address this hypothesis, the *MR* -2G/C and I180V SNPs were examined for association with the 1-hour cortisol awakening response in a large cohort of patients with a lifetime diagnosis of MDD (remitted and current). Subsequently, data were stratified for sex to assess sex-dependent effects. Finally, possible interaction effects with the MR were tested for stressful life events and frequent use of SSRIs.

Methods

Study population

Data were used from the Netherlands Study of Depression and Anxiety (NESDA), an eight-year longitudinal cohort study on the causes and course of depressive and anxiety disorders in people aged 18-65 years. For the NESDA study, a total of 2981 respondents were recruited from the general population and from primary care and specialized mental health care practices, including 2329 patients with a lifetime depressive and/or anxiety disorder and 652 subjects without any (lifetime or current) depressive and/or anxiety disorder. Among those subjects a primary clinical diagnosis of psychotic disorder, obsessive-compulsive disorder, bipolar disorder, or severe addiction disorder and not being fluent in Dutch was excluded. All participants provided written informed consent before inclusion. For details on the NESDA study see (Penninx et al., 2008).

In the present study patients were selected when they had a lifetime MDD diagnosis (n= 1925), as assessed with the DSM-IV Composite International Diagnostic Interview (CIDI) version 2.1. Patients were excluded when they indicated not to be from western European ancestry (n= 109), when taking corticosteroids (n= 15) or when pregnant or breastfeeding (n= 11). Of this subset of 1790 MDD patients, genotypes were available for 1572 individuals, which were assessed earlier as part of a large genome wide association (GWA) study for MDD, the GAIN-MDD study (Sullivan et al., 2009). Saliva cortisol data were available for 1091 of the 1572 genotyped MDD patients. When comparing this group of 1091 respondents with the subjects for whom no genotypes or saliva data were available (n= 699), they did not differ in sex. However, they were slightly older (43.6 ± 12.4 vs. 39.6 ± 12.2; $p< .001$), were more educated (12.2 ± 3.2 yrs vs. 11.6 ± 3.2 yrs; $p< .001$) and were more often currently depressed (54.3% vs. 45.7%; $p< .01$). Finally, an additional group of 65 individuals was excluded because less than 2 valid CAR measurement points were available, leaving a final group of 1026 respondents. Of this final group of 1026 lifetime MDD patients, 555 (54.1%) had a current depression (depression diagnosis in the past 6 months) and 715 (69.7%) had a comorbid lifetime anxiety disorder. The present study combines remitted and current depressed patients as the previous analysis by Vreeburg et al. (Vreeburg et al., 2009a) showed that the CAR was similarly heightened in both groups when compared to the controls.

Sociodemographic, sampling and health factors

Covariates

Multiple sociodemographic, sampling and health factors that were previously taken along as (possible) determinants of salivary cortisol were considered as potential covariates in the present study (Vreeburg et al., 2009a). These include: sex (1= men; 2= women), age (in years), education (years of attained education), time of awakening on sampling day, working on sampling day (0= not working; 1= working), sampling on a weekday vs. weekend day (0= weekend day; 1= weekday), season (0= dark months, that is October through February; 1= months with more daylight, that is March through September), average sleep duration during the last 4 weeks (0= more than 6 h sleep a night; 1= 6 h of sleep or less a night), smoking status (0= no current smoker; 1= current smoker) and physical activity (which was assessed using the International Physical Activity Questionnaire and expressed as activity per 1000 MET-minutes, a metabolic equivalent of the number of calories spent per minute, per week).

Potential moderators of genetic association

Based on literature, potential interaction effects with the *MR* gene were tested for sex (Carey et al., 1995; Turner, 1997; Kumsta et al., 2007; van Leeuwen et al., 2010a), SSRIs (0= no frequent SSRI use; 1= frequent SSRI use; for at least 1 month) (Seckl and Fink, 1992; Bjartmar et al., 2000; Otte et al., 2010) and stress (Gesing et al., 2001; Bet et al., 2009), i.e. childhood trauma before age 16 (index score on the Netherlands Mental Health Survey and Incidence Study childhood trauma interview (de Graaf et al., 2004) assessing the frequency of emotional neglect, psychological neglect, physical abuse and sexual abuse experienced before the age of 16 years; median split, 0= no or infrequent trauma; 1= frequent trauma) and number of life events in the past year (including illness or death of a

family member among others; median split, 0= no life events; 1= 1+ life events). Multiple studies suggest an interaction between the *MR* gene and SSRIs or TCAs (Seckl and Fink, 1992; Holsboer and Barden, 1996; Holsboer, 1999; Bjartmar et al., 2000; Otte et al., 2010). Due to the low number of cases using TCAs (n= 35) or other antidepressants that may modulate MR activity, an interaction effect with the *MR* could not be tested. Because of potential differential mechanisms we did not initially choose to test for an interaction effect between the *MR* and all antidepressants (benzodiazepines not included) combined.

Salivary cortisol measurements

At the baseline interview, the patients were instructed to collect saliva samples using salivettes (Sarstedt AG and Co, Nümbrecht, Germany) at home and on a regular (preferably working) day shortly after the interview. This is a minimally intrusive method to assess the free and active form of cortisol that has previously been shown to be a reliable measure of free cortisol in the blood (Kirschbaum and Hellhammer, 1994). Patients were instructed not to eat, drink, smoke or brush their teeth within the 15 min before sampling. The CAR was measured at 4 time points: at awakening (T1) and at 30 (T2), 45 (T3) and 60 (T4) minutes after awakening. Participants were instructed to store the salivettes in their refrigerator until returning them by mail. For details on cortisol measurements, see (Vreeburg et al., 2009a). In short, cortisol analysis was performed by competitive electrochemiluminescence immunoassay (E170; Roche, Basel, Switzerland). The functional detection limit was 0.07 µg/dL or 2 nMol/L and the intra-assay and inter-assay variability coefficients were below 10%.

Cortisol awakening response (CAR)

For genetic association analyses with the course of the CAR, at least 2 valid CAR measurement points had to be available, that is when collected within a margin of 5 min before or after the protocol time and when values were not more than 2 standard deviations (SDs) from the mean. With Linear Mixed Model (LMM) analyses missing values could be interpolated, which was conducted for 24 subjects with 2 CAR measurement points and 96 subjects with 3 CAR measurement points. For the remaining 906 subjects all 4 data points were available. Besides studying the course of the CAR with LMM analysis, also the area under the curve (AUC) with respect to the increase (AUCi) and with respect to the ground (AUCg) were used, calculated according to the formulas by (Pruessner et al., 2003). The AUCg is a measure for the total cortisol secretion during the first hour after awakening, while the AUCi is a measure for cortisol increase with respect to awakening (T0) and therefore is a measure of the dynamics of the CAR (Clow et al., 2004). For association analyses with the AUC subjects were included when all 4 1-hour awakening cortisol samples were available (n= 906).

Genotyping

Genotyping of the patients was performed as part of a large GWA study, the GAIN-MDD study (Sullivan et al., 2009). Details on blood sampling and data collection can be found elsewhere (Boomsma et al., 2008). Individual genotyping was conducted by using the Perlegen GWAS platform (Mountain View, CA, USA). The SNPs that were present on these arrays were selected to tag common variation in the HapMap European and Asian

populations. For the *MR* gene the two common and functional *MR* -2G/C (rs2070951_GC) and I180V (rs5522_AG) SNPs were present. Based on DNA sequencing and haplotype reconstruction by our group it is known that, in the Dutch population, these two SNPs tag the three most common haplotypes located in exon 2 and extending into the promoter region (**Chapter 3**).

Statistical analyses

Allele frequencies for the different SNPs were tested for Hardy-Weinberg equilibrium (HWE) using HaploView (version 4.1 for Mac OSX; 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. Individual haplotypes were reconstructed with SNPHAP (version 1.3; available online at http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt). Further analysis was performed in SPSS, version 16.0 for Mac OSX (SPSS Inc., Chicago, IL, USA).

Differences between men and women for the various characteristics were verified using an independent-samples t-test, a Mann-Whitney test or a χ^2-test. Before testing for sex differences, a square root transformation was used to reach a normal distribution for awakening time and physical activity. The 4 morning cortisol measures were positively skewed and therefore log-transformed data were used in Linear Mixed Models (LMM) analysis, for the AUCg and AUCi non-transformed values could be used. For the data shown in Figure 1 values were back-transformed.

First, associations between the single *MR* SNPs and AUCg or AUCi as outcome variables were tested with AN(C)OVA. Linear regression analysis was used to analyze associations between *MR* haplotypes and the AUCg or AUCi. Putative covariates were entered first, followed by adding the haplotypes in the second step. Random coefficient analysis of the 4 morning cortisol values was conducted with the help of LMM analysis. This method can interpolate missing values and it keeps the correlation between repeated data into account (Gueorguieva and Krystal, 2004). The model included a random intercept, taking into account different intercepts for the different subjects, the SNPs or haplotypes, time points (T1, T2, T3 or T4) and all covariates were entered in the model as fixed factors. To examine whether the different genetic variants affected the course of cortisol levels after awakening we added a variant-by-time interaction term. Second, because of clear sex-dependent effects of *MR* (and *GR*) gene variants in earlier studies, interaction effects between the SNPs and sex were verified and association analysis was repeated in both sex strata (Kumsta et al., 2007; van Leeuwen et al., 2010a). Third, an interaction effect was tested for the *MR* SNPs with SSRIs or stress, i.e. childhood trauma or recent life events. Due to low frequencies, no interaction effect could be tested for use of TCAs (n= 35). A two-sided *p*-value below .05 was considered statistically significant. For significant findings effect sizes are given as $r = \sqrt{(t^2 / t^2 + df)}$. Our main interest was to determine the association between the *MR* -2G/C SNP and the CAR. Because of multiple testing a Bonferroni correction was applied where appropriate.

Results

Population characteristics

Characteristics of the 1026 subjects are presented in **Table 1**. The mean age of this subpopulation was 43.5 years (SD= 12.3, range 18 — 65) and 68.4% was female. Of the 1026 subjects 72.3% showed an increase in cortisol level in the first hour after awakening. The two sexes differed significantly in age, education level, smoking behavior, sleep duration, current depression diagnosis and cortisol level at T2 and T4. No significant differences in demographics were found depending on the *MR* SNP genotypes or haplotypes.

Table 1 Sample characteristics of the total group and comparisons between men and women

Variable	Total n	Total group n= 1026	Men n= 324 (31.6%)	Women n= 702 (68.4%)	*p*-value
Age, mean (SD), y	1026	43.5 (12.3)	45.3 (11.2)	42.6 (12.8)	**.001**
Education level, mean (SD), y	1026	12.2 (3.2)	11.9 (3.1)	12.4 (3.2)	**.02**
Smoking, %	1026	36.7	41.0	34.8	**.05**
Physical activity, mean (SD)	1026	3.7 (3.1)	3.7 (3.2)	3.7 (3.0)	.79
Sampling factor					
Time of awakening, mean (SD)	1026	07:31 (1 h, 13 min)	07:30 (1 h, 12 min)	07:31 (1 h, 13 min)	.71
Working on day of sampling, %	1026	57.5	59.9	56.4	.30
Sampling on a weekday, %	1026	91.5	89.2	92.6	.07
Sampling in month with more daylight, %	1026	58.0	58.3	57.8	.88
≤ 6 h of sleep, %	1026	29.5	34.0	27.5	**.04**
Frequent antidepressant use					
TCA, %	1026	3.4	3.4	3.4	.98
SSRI, %	1026	22.1	23.5	21.5	.49
Other, %	1026	7.8	9.3	7.1	.24
Benzodiazepines, %	1026	8.6	9.9	8.0	.31
Trauma					
Childhood trauma index score, regularly, %	1022	48.5	45.5	49.9	.19
Life events in past year, 1 or more events, %	1026	39.1	35.5	40.7	.11
Depression					
Current, %	1026	54.1	59.6	51.6	**.02**
Comorbid anxiety disorder, %	1026	69.7	67.6	70.7	.33
Cortisol					
CAR, mean (SD), nMol/L					
T1, at awakening	1014	17.0 (6.8)	17.8 (7.5)	16.7 (6.4)	.07
T2, 30 min after awakening	1005	21.4 (9.3)	22.6 (10.9)	20.9 (8.5)	**.03**
T3, 45 min after awakening	1000	20.2 (9.8)	20.7 (11.5)	20.1 (9.0)	.88
T4, 60 min after awakening	1011	18.0 (9.7)	16.9 (8.1)	18.5 (10.3)	**.03**
AUCg, mean (SD), nMol/L/h	906	19.6 (7.1)	20.2 (7.7)	19.3 (6.8)	.10
AUCi, mean (SD), nMol/L/h	906	2.5 (6.3)	2.2 (7.0)	2.6 (5.9)	.31
MR variants					
rs2070951 (-2) GG/CG/CC, freq.	1026	.23 / .54 / .23	.21 / .54 / .25	.24 / .54 / .22	.30
rs5522 (I180V) AA/GA/GG, freq.	1026	.78 / .20/ .02	.75 / .23 / .02	.79 / .19 / .02	.30
MR hap 1 -2G/180A, freq.	1026	.50	.48	.52	
MR hap 2 -2C/180A, freq.	1026	.38	.39	.37	.23
MR hap 3 -2C/180G, freq.	1026	.12	.14	.11	

Note: significant *p*-values are indicated in bold.

Abbreviations: SD= standard deviation; MET= metabolic energy turnover; TCA= tricyclic antidepressant; SSRI= serotonin transporter reuptake inhibitor; CAR= cortisol awakening response; AUCg = area under the morning curve with respect to the ground (=(((T1 + T2)/2)*0.5) + (((T2 + T3)/2)*0.25) + (((T3 + T4)/2)*0.25)); AUCi = area under the morning curve with respect to the increase = (((T1 + T2)/2)*0.5) + (((T2 + T3)/2)*0.25) + (((T3 + T4)/2)*0.25)) - (T1*(0.5 + 0.25 + 0.25)) (Pruessner et al., 2003).

Genotype and haplotype frequencies

Allele frequencies of the *MR* SNPs were in HWE, as assessed using HaploView. Frequencies for the *MR* -2G/C and I180V genotypes and haplotypes (**Table 1**) and the inter-marker LD scores (*D'*= 1.0; r^2= .14) were similar as previously described (Derijk, 2009; van Leeuwen et al., 2010a; van Leeuwen et al., 2011) (Chapter 3 till 5). Concordant with previous results, three main haplotypes were found; haplotype 1 consisting of the -2 G-allele and the 180 I-allele (or A nucleotide; hap 1 freq.= .50); haplotype 2 consisting of the -2 C-allele and the 180 I-allele (hap 2 freq.= .38) and haplotype 3 consisting of the -2 C-allele and the 180 V-allele (or G nucleotide, hap 3 freq.= .12). Notably, there were no individuals carrying a haplotype consisting of the G-allele of the -2G/C SNP combined with the V-allele (or G nucleotide) of the I180V SNP, in accordance with our previous observations this combination is very rare.

Associations between *MR* gene variants and the CAR

Of the variables listed in **Table 1** age, smoking, time of awakening, working on day of sampling and frequent TCA use were significant determinants of the CAR in the total group or in the women or men separately. Without or with adjustment for these covariates (except for TCAs, due to the small number; n= 35) no effect was found for the -2G/C and I180V SNPs on the CAR in the total group. However, a significant interaction effect was found for the -2G/C SNP with sex on the AUCi (*p*= .01) and a trend was found for an interaction effect on the AUCg (*p*= .08). Therefore, for further analysis data were stratified for sex. The course of the CAR over time was slightly modulated by the *MR* -2G/C SNP only in women, as reflected by a trend for an interaction effect with time (*p*= .06; **Figure 1A**) and/or an attenuated cortisol increase after awakening (AUCi) in carriers of the -2 C/C genotype (*p*= .03; **Table 2**). No effect was observed for the total morning cortisol secretion, i.e. no significant association with the AUCg and/or no direct SNP effect in LMM analysis, only in men a trend was found for a lower AUCg in -2 C-allele carriers (*p*= .06). In addition, no effect was observed for the I180V SNP, not in men or women. In women both the haplotypes 2 and 3 lowered (significant or trend) the AUCi compared to haplotype 1, explaining however only 0.9% of the variance.

As a third step, interaction was verified with frequent use of SSRIs. For the AUCi a trend for a three-way (-2G/C-by-sex-by-SSRI) interaction effect was found (*p*= .06; for AUCg *p*= .49). In addition, the effect of the -2G/C SNP on the CAR in women was found to be due to an interaction with SSRI use (*p*= .07 for the AUCg and *p*= .05 for the AUCi). No significant interaction effect was found in men (*p*> .30) or in the total group (men and women; *p*> .10). Interestingly, subsequent stratification of the data for the use of SSRIs (**Figures 1B** and **C**) showed that the *MR* -2G/C SNP was associated with variability in the CAR only in the individuals (both men and women) using SSRIs (n= 227, of whom 149 had a current MDD diagnosis). In the female SSRI users the -2G/C SNP clearly affected the course of the CAR throughout time, SNP-by-time interaction *p*= .006 (after a Bonferroni correction for six tests, giving a new significance threshold of *p*= .05/6= .008, this is still significant; effect size *r* for AUCi: CC vs. GG *r*= .27, *p*< .01; CC vs. GC *r*= .27, *p*< .01). The -2G/C SNP also had a direct effect on total morning cortisol secretion (*p*= .03 in LMM analysis; AUCg: CC vs. GG *r*= .23, *p*= .01; CC vs. GC *r*= .14, *p*= .11). In the male SSRI users only a direct effect on total

cortisol secretion was observed (p= .02 in LMM analysis; AUCg: CC vs. GG r= .21, p= .11; CC vs. GC r= .18, p= .16). Notably, among the SSRI users the CAR was entirely blunted in female -2C/C carriers and was highest in male and female -2G/G carriers.

Table 2 Unadjusted and adjusted area under the curve cortisol values according to *MR* SNPs and haplotypes, *F*-statistics, regression coefficients (*B*) and *p*-values

		rs2070951			rs5522		*MR* haplotype 1-3		
		GG	GC	CC	AA	AG/GG	Constant	Hap 2	Hap 3
Women	**AUCg**, mean (SD)	19.4 (6.5)	19.4 (6.8)	19.0 (7.2)	19.5 (6.9)	18.4 (6.3)	19.5 (0.5)	19.5 (0.4)	18.5 (0.6)
(n= 624)	Unadjusted	*F (1, 621)= 0.33; p= .56*			*F (1, 622)= 2.77; p= .10*		ref.	*B*= -0.00 (0.43); *p*= 1.0	*B*= -0.99 (0.63); *p*= .12
	Adjusted	*F (2, 617)= 0.34; p= .71*			*F (1, 618)= 2.29; p= .13*		ref.	*B*= -0.10 (0.42); *p*= .80	*B*= -0.94 (0.61); *p*= .13
	Adjusted, no SSRI use	*F (2, 489)= 0.07; p= .93*			*F (1, 490)= 2.13; p= .15*		ref.	*B*= 0.45 (0.48); *p*= .35	*B*= -0.73 (0.68); *p*= .29
	Adjusted, SSRI users	*F (2, 121)= 3.55;* ***p= .03***			*F (1, 122)= 0.04; p= .84*		ref.	*B*= -2.27 (0.83); ***p< .01***	*B*= -1.33 (1.37); *p*= .33
Men	**AUCg**, mean (SD)	22.0 (9.1)	19.2 (7.0)	20.6 (7.6)	20.5 (7.9)	19.3 (7.3)	20.9 (0.8)	20.4 (0.7)	19.6 (1.0)
(n=282)	Unadjusted	*F (1, 279)= 0.99; p= .32*			*F (1, 280)= 1.34; p= .25*		ref.	*B*= -0.44 (0.72); *p*= .54	*B*= -1.30 (1.01); *p*= .20
	Adjusted	*F (2, 275)= 2.86; p= .06*			*F (1, 376)= 0.84; p= .36*		ref.	*B*= -0.09 (0.71); *p*= .89	*B*= -0.89 (1.00); *p*= .38
	Adjusted, no SSRI use	*F (2, 214)= 0.99; p= .37*			*F (1, 215)= 0.79; p= .38*		ref.	*B*= 0.11 (0.81); *p*= .89	*B*= -1.03 (1.17); *p*= .38
	Adjusted, SSRI users	*F (2, 54)= 4.35;* ***p= .02***			*F (1, 55)= 0.13; p= .73*		ref.	*B*= -1.18 (1.51); *p*= .44	*B*= -1.24 (2.04); *p*= .54
Women	**AUCi**, mean (SD)	3.1 (6.0)	2.9 (5.6)	1.5 (6.4)	2.8 (6.0)	1.9 (5.4)	3.4 (0.4)	2.7 (0.4)	2.3 (0.6)
(n= 624)	Unadjusted	*F (1, 621)= 4.88;* ***p= .03***			*F (1, 622)= 2.18; p= .14*		ref.	*B*= -0.70 (0.38); *p*= .06	*B*= -1.03 (0.55); *p*= .06
	Adjusted	*F (2, 617)= 3.60;* ***p= .03***			*F (1, 618)= 1.94; p= .16*		ref.	*B*= -0.77 (0.37); ***p= .04***	*B*= -1.03 (0.54); *p*= .06
	Adjusted, no SSRI use	*F (2, 489)= 0.86; p= .42*			*F (1, 490)= 2.23; p= .14*		ref.	*B*= -0.35 (0.42); *p*= .41	*B*= -0.91 (0.59); *p*= .13
	Adjusted, SSRI users	*F (2, 121)= 6.31;* ***p< .01***			*F (1, 122)= 0.00; p= 1.0*		ref.	*B*= -2.63 (0.79); ***p= .001***	*B*= -1.27 (1.31); *p*= .34
Men	**AUCi**, mean (SD)	2.8 (8.9)	1.5 (5.5)	3.1 (7.8)	2.3 (7.2)	1.8 (6.3)	2.0 (0.8)	2.2 (0.7)	2.0 (0.9)
(n= 282)	Unadjusted	*F (1, 279)= 0.11; p= .74*			*F (1, 280)= 0.31; p= .58*		ref.	*B*= 0.28 (0.65); *p*= .67	*B*= -0.02 (0.92); *p*= .99
	Adjusted	*F (2, 275)= 1.74; p= .18*			*F (1, 276)= 0.90; p= .77*		ref.	*B*= 0.39 (0.65); *p*= .55	*B*= 0.26 (0.93); *p*= .78
	Adjusted, no SSRI use	*F (2, 214)= 0.76; p= .47*			*F (1, 215)= 0.02; p= .89*		ref.	*B*= -0.21 (0.74); *p*= .77	*B*= -0.25 (1.06); *p*= .82
	Adjusted, SSRI users	*F (2, 54)= 1.92; p= .16*			*F (1, 55)= 1.03; p= .31*		ref.	*B*= 2.89 (1.52); *p*= .06	*B*= 0.63(2.06); *p*= .76

Notes: significant *p*-values are indicated in bold. Adjusted= adjusted for age, smoking, awakening time, working on day of sampling and lifetime diagnosis of major depressive disorder.
Abbreviations: AUCg = area under the morning curve with respect to the ground; AUCi = area under the morning curve with respect to the increase: SD= standard deviation; SSRI= serotonin transporter reuptake inhibitor.

Additional correction for remitted vs. current depression did not change the results. LMM analysis in only the 906 subjects with all 4 CAR data points available gave similar (slightly stronger) results. In addition, results did not change after excluding the subjects taking TCAs (n= 35; of the subjects using SSRIs, n= 227, only 2 were also taking TCAs). An interaction effect between the *MR* SNP and the use/no use of all antidepressants combined was verified but was not significant. No interaction effect was found between the *MR* -2G/C SNP and childhood trauma or recent life events. Finally, as earlier studies indicate that sex hormones can effect MR (and GR) mRNA and protein expression and protein binding (Carey et al., 1995; Turner, 1997), a possible interaction was verified between the -2G/C SNP and the use of oral contraceptives (OC) or menstrual phase, however, no significant interaction was observed.

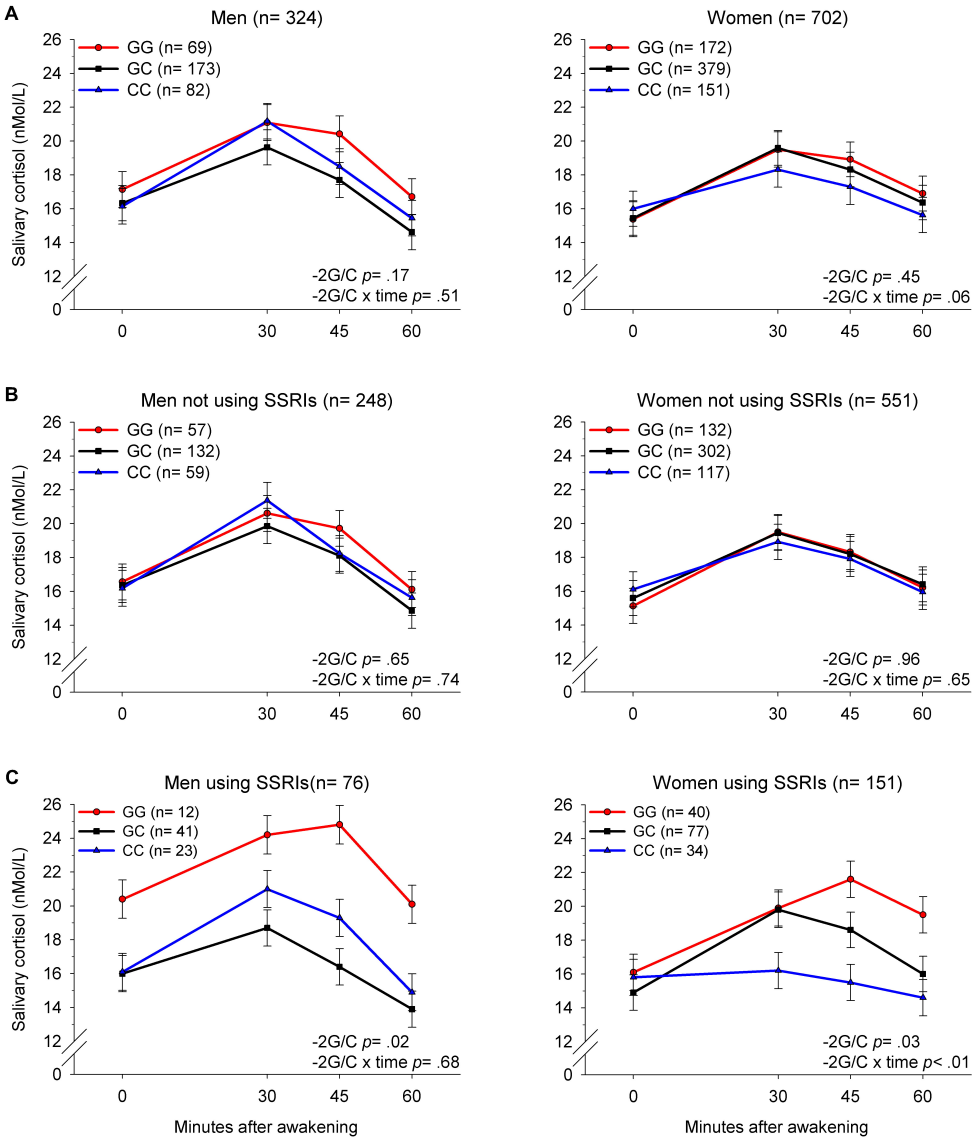

Figure 1 Mean cortisol awakening response levels adjusted for age, smoking, awakening time and working on day of sampling. Error bars represent standard errors.

Discussion

This study shows that the *MR* -2G/C SNP modulates the CAR in lifetime MDD patients depending on the use of SSRIs; a clear effect of the *MR* -2G/C SNP was found specifically in subjects (men and women) frequently using SSRIs. No effect of the *MR* SNPs on the CAR was found in subjects not using SSRIs. The results, therefore, suggest that *MR* gene

variants can have substantial effects on HPA axis activity while interacting with other factors like use of SSRIs.

The current results are partly in line with a first report revealing that the *MR* -2 C-allele significantly associated with slightly lower morning cortisol levels among an elderly cohort consisting for 66% of women (Kuningas et al., 2007). Of note is that these results were based on a single morning blood sample for which no effect of time of awakening was taken into account. Earlier studies showed that cortisol levels measured at multiple time points in the morning are more reliable (Pruessner et al., 1997). The present results are also partly in line with a more recent study by our group. Among a group of healthy individuals (n= 218) the CAR was lower in subjects with the *MR* -2C/C genotype (van Leeuwen et al., 2010a). However, this association was not significant and was found only in men (n= 93) and not in women (n= 125; genotype-by-sex effect *p*= .20). Together the results indicate that the *MR* -2 C-allele is related to a decrease in cortisol levels under specific conditions.

Since the MR is involved in tonic inhibition of basal corticosteroid levels, an increased expression of the MR protein is expected to result in lower cortisol levels. In accordance with this hypothesis and the above mentioned results, in cell lines the -2 C-allele results in increased expression of the MR protein, resulting in a higher capacity to activate target genes (van Leeuwen et al., 2010a, 2010b). The -2G/C variant interferes with expression of the MR protein potentially at the translational level. Notably, MR expression is highly dynamic. Following exercise or an acute single psychological stressor, but also during ageing changes in MR expression can be observed, at least in the latter two conditions associated with changes in HPA axis reactivity (van Eekelen et al., 1991; Gesing et al., 2001; Chang et al., 2008). Based on the present and previous association studies (DeRijk et al., 2006; van Leeuwen et al., 2010a, 2011) we hypothesize that only under challenging conditions (like stress or medication) the *MR* gene variants may affect HPA axis activity. Here, a clear effect of the *MR* -2G/C SNP was found only in the lifetime MDD patients frequently using SSRIs. Among those subjects, carriers of the *MR* -2 C-allele showed an attenuated CAR, with a clear allele-dose effect only in women. On the other hand, carriers (men and women) of the -2G/G genotype showed the highest CAR, with elevated cortisol levels even 60 min after awakening. In the previous study by van Leeuwen et al. (van Leeuwen et al., 2010a) also a more distinct effect of the -2G/C SNP on the CAR was detected following pre-treatment with dexamethasone and in a sex-dependent manner. Finally, a significant effect of *MR* gene variants on ACTH, cortisol and heartbeat could be observed under psychosocial stress conditions (DeRijk et al., 2006; van Leeuwen et al., 2011).

Importantly, the two functional *MR* SNPs described here are linked to multiple SNPs located in the *MR* gene promoter region. These promoter SNPs result in turn in differences in transcriptional activity, leading to differential mRNA and protein regulation (**Chapter 3**). Together the SNPs result in three major haplotypes (which are tagged by the -2G/C and I180V SNPs) with distinct genetic sequences, which may modulate MR expression and HPA activity in a context-dependent manner. Most likely, these SNPs located in the promoter region modulate effects of other factors like corticosteroids, sex steroids or antidepressants,

leading to gene-variant specific changes in MR regulation. Proof for possible interactions between the *MR* gene and sex steroids has been demonstrated for both estrogens and progesterone, which modulate mRNA and/or protein expression and binding of corticosteroid receptors (Carey et al., 1995; Turner, 1997). This could provide an explanation for the gender-dependent effects of the MR on the CAR.

Multiple indications for an interaction between MR signaling and the serotonin system exist. Changes in hippocampal MR expression in mice influence expression of the serotonin receptor 1A (5-HT1A) (Rozeboom et al., 2007). Moreover, the MR, GR and 5-HT1A receptors are co-expressed in specific cells of the hippocampus, while the level of MR occupation by cortisol affects the 5-HT1A-receptor mediated hyperpolarization response (Joels and Van Riel, 2004). On the other hand, serotonin but also SSRIs increase MR and/or GR expression *in vivo* and *in vitro* (Seckl and Fink, 1991, 1992; Robertson et al., 2005). Possibly, SSRIs affect MR expression directly or indirectly through 5-HT in a genotype-dependent manner, eventually leading to differential cortisol regulation.

Several lines of evidence suggest a role for the MR in the CAR. Highest MR mRNA expression levels have been measured in the human hippocampus, while much lower levels were detectable in other areas such as the amygdala, prefrontal cortex and anterior cingulate cortex (**Chapter 2**). A putative role for the hippocampus in the regulation of the CAR was previously demonstrated (Buchanan et al., 2004). In addition, the CAR was recently postulated to enable individuals to anticipate upcoming daily events, a process in which the hippocampus is central and in which the MR is involved (de Kloet et al., 2005; Fries et al., 2009). Moreover, the hippocampus is important for tonic inhibition of the HPA axis, which is MR mediated. Taken together, the data fit with a role of the MR, predominantly located in the hippocampus, in the control of the CAR.

The function and importance of the CAR for health and disease is still unclear. However, data indicate that small differences in the CAR can be of clinical relevance as they are associated with physiological and psychological disturbances (Fries et al., 2009; Vreeburg et al., 2009a). It was demonstrated that the CAR was elevated not only in current depressed patients but also in remitted depressed patients and in unaffected subjects with a parental history of depression or anxiety disorder, as assessed with the DSM-IV Composite International Diagnostic Interview (CIDI) (Vreeburg et al., 2009a, 2010a). This suggests that an increased CAR in MDD patients is not only a state marker but represents in part a trait. Here, we identified a biological determinant of inter-individual variability in the CAR, possibly representing a vulnerability/protective factor for the pathophysiology or course of depressed mood. Moreover, the *MR* gene variants may underlie in part the development of particular symptoms of depression, not only problems with mood but also for example cognitive problems (Kuningas et al., 2007). Indeed, multiple studies suggest that MR activity influences cognitive flexibility in healthy individuals (Otte et al., 2007; Schwabe et al., 2009).

Normalization of the HPA axis, either by alleviation of hypercortisolism or a decrease of reactivity as measured by the Dex-CRH test, is predictive for clinical benefit (Barden et al., 1995; Zobel et al., 2004). In the present study, the SSRIs by themselves had no effect on

the CAR. However, the *MR*-by-SSRI interaction effect on the CAR was remarkably distinct; depending on *MR* genotype, 25 percent of the women and men using SSRIs showed a small or even flattened CAR (-2 C-allele carriers), while another 25 percent of the patients (-2G/G carriers) frequently using SSRIs displayed a high CAR compared to the other genotype groups. This effect could indicate that some patients benefit from SSRI treatment when it comes to neuroendocrine normalization, while others experience deterioration depending on their *MR* genotype. A role of the MR in pharmacological treatment of depression was recently demonstrated in a study by Otte et al. (Otte et al., 2010), in which administration of a MR agonist accelerated the response of MDD patients to the SSRI escitalopram. The results complement the results of earlier studies showing that the MR antagonist spironolactone hampers the response to the TCA amitriptyline (Holsboer, 1999). It is plausible that these effects are also depending on *MR* genetic makeup.

There are some limitations to this study. The number of men that was included in this study was much smaller than for women. Therefore it was more difficult to draw conclusions based on the effects of *MR* variants on the CAR seen in the men, especially for the men using SSRIs. In addition, the CAR was assessed only once. Although saliva cortisol levels show high intra-individual stability, there might still be some day-to-day variation, which could influence associations found for the *MR* gene. Finally, although we corrected our associations for multiple confounders, we cannot exclude the possibility of any other residual confounding factor not evaluated here, like cognitive flexibility or appraisal, which also appears to be influenced by the MR (Otte et al., 2007; Schwabe et al., 2009). The results presented here need to be replicated, including a larger number of subjects taking antidepressants like SSRIs and TCAs. Moreover, future studies should assess the effect of *MR* genotype on SSRI efficacy on clinical outcome in MDD patients.

To conclude, we have identified the MR as a possible modulator of the CAR in depressed patients. A clear effect of the functional *MR* -2G/C SNP on the CAR was found in the lifetime MDD patients frequently using SSRIs, with highest early morning cortisol levels observed in *MR* -2G/G carriers and lower levels in -2 C-allele carriers. No effect was found in patients not using SSRIs. The finding of a *MR* genotype-by-SSRI interaction effect on the dynamics of the CAR could be of importance for future therapy selection and for development of novel pharmacological treatments.

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