Mineralocorticoid receptor in human brain : a key player in resilience

Klok, M.D.

Part 1

Molecular studies

Our first step was to find evidence for MR disregulation in depression. Therefore, MR and GR expression patterns in the *postmortem* human brain were assessed and compared between depressed patients and non-depressed subjects (**Chapter 2**).

To further study the role of the MR in human neuroendocrine regulation, behavior and psychopathology, we aimed to identify functional *MR* gene variants that are subsequently used as tools in genetic association studies. The *MR* gene promoter region was analyzed for the presence of SNPs that exist besides the known and functional -2G/C and I180V SNPs. The *MR* gene promoter SNPs and the associated haplotypes were tested for functionality in cultured cells (**Chapter 3**).

Chapter 2

Decreased expression of mineralocorticoid receptor mRNA and its splice variants in postmortem brain regions of patients with major depressive disorder

Melanie D. Klok, Simone R. Alt, Alicia J.M. Irurzun Lafitte, Jonathan D. Turner, Egbert A.J.F. Lakke, Inge Huitinga, Claude P. Muller, Frans G. Zitman, E. Ronald de Kloet, Roel H. DeRijk

Journal of Psychiatric Research 2011; 45 (7): 871-878.
See also the supplemental data at the end of this chapter.

Abstract

Background: Appropriate signaling in the brain by the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) is critical in regulation of the hypothalamic-pituitary-adrenal (HPA) axis, emotional arousal and cognitive performance. To date, few data exist on MR (and GR) expression in the brain of patients suffering from major depressive disorder (MDD).
Methods: With the help of quantitative PCR we assessed MR and GR mRNA expression, including the splice variants MRα and MRβ, in tissue samples from the hippocampus, amygdala, inferior frontal gyrus, cingulate gyrus and nucleus accumbens. Expression levels were compared between tissue samples from 6 MDD patients and 6 non-depressed subjects.
Results: Relative to total GR, total MR mRNA expression was higher in hippocampus and lower in the amygdala, inferior frontal gyrus and nucleus accumbens. Both MRα and MRβ could be detected in all brain regions that were analyzed, although MRβ expression was low. Significantly lower expression levels (30–50%) were detected for MR or GR in hippocampal, inferior frontal gyrus and cingulate gyrus tissue from MDD patients ($p < .05$), while no differences were found in amygdala or nucleus accumbens.
Conclusions: The data show that both MRα and MRβ mRNA are expressed throughout the human limbic brain with highest expressions in the hippocampus. A decreased expression of corticosteroid receptors in specific brain regions of MDD patients could underlie HPA hyperactivity, mood and cognitive disturbances often observed in patients suffering from stress-related psychopathologies.

Keywords: mineralocorticoid receptor, mRNA expression, splice variants, brain, major depressive disorder

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system central in maintaining homeostasis and adaptation during challenges (de Kloet et al., 2005). Its end hormone cortisol binds to two related receptor types, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet, 1985). Due to its low affinity the GR only becomes activated during ultradian, circadian or stress peak cortisol levels and mediates negative feedback action on the HPA axis. In peripheral tissues the GR plays a crucial role in immunity, metabolism and cardiovascular control (DeRijk et al., 2002), while in brain the GR mediates learning and memory processes (Oitzl and de Kloet, 1992).

The MR is classically known for mediating maintenance of salt- and water balance in the kidney (Funder, 2005). The kidney MR is selective for aldosterone because cortisol is inactivated by 11β-hydrosteroid dehydrogenase type 2 (11βHSD2). In the brain the MR is abundantly expressed in limbic structures. Because of the excess in circulating cortisol this naturally occurring glucocorticoid rather than aldosterone is the favorite ligand for the limbic brain MR, which binds cortisol with a 10-fold higher affinity than the GR (Reul and de Kloet, 1985). Even at rest the MR in limbic brain structures is substantially occupied by cortisol and is thought to mediate tonic inhibition and reactivity of the HPA axis. Together with the newly identified membrane variant (Karst et al., 2005; Joels et al., 2008; Karst et al., 2010) the brain MR seems involved in the appraisal of a novel situation and modulates behavioral flexibility (Oitzl et al., 1994; Berger et al., 2006; Otte et al., 2007). Moreover, the MR is important for cognitive processes such as selective attention (Otte et al., 2007) and emotions like anxiety (Brinks et al., 2007b; Rozeboom et al., 2007).

Many patients with a depressive and/or anxiety disorder show disturbances in HPA axis reactivity, cognitive impairments and emotional disregulations (Holsboer, 2001; Nemeroff et al., 2006). These observations may suggest a role for inappropriate MR and/or GR activity and several lines of research confirm this. Firstly, animal models of depression suggest the implication of deficits in corticosteroid receptor signaling (Gass et al., 2001). Secondly, patients with affective disorders often show non-suppression of cortisol after dexamethasone (Holsboer, 2001), which is indicative for dysfunctioning of central corticosteroid receptors, especially the GR. Thirdly, administration of the MR antagonist spironolactone to depressed patients revealed aberrant MR-mediated HPA regulation (Young et al., 2003), while the clinical response to antidepressants is influenced by modulation of MR activity (Holsboer, 1999; Otte et al., 2010). Antidepressants are known to induce hippocampal MR and GR expression (Seckl and Fink, 1992; Lopez et al., 1998; Bjartmar et al., 2000), while diurnal HPA hyperactivity is normalized preceding clinical relief after long-term antidepressant treatment in depressed patients (Barden et al., 1995; Zobel et al., 2004). Together with other findings these data have lead to the hypothesis that an imbalance in MR and GR functioning may be a risk factor for psychopathology (de Kloet et al., 1998).

Here we report data on the expression of MR and GR in *postmortem* brain regions of patients suffering from major depressive disorder (MDD) and controls. A recent report (Alt et

al., 2010) presented results of a thorough investigation of GR mRNA expression in tissue of 5 human *postmortem* brain regions isolated from 6 MDD patients and 6 non-depressed subjects. Total GR mRNA levels were comparable between both groups (MDD patients showed a small but non-significant decrease), while expression of multiple GR promoter splice variants was significantly different. Here we focus on MR expression. While in the rodent brain MR expression has been detected mainly in the hippocampus (de Kloet and Reul, 1987), in non-human primate brain MR expression turns out to be more widespread (Patel et al., 2000; Sanchez et al., 2000), with also high expressions in for example the superficial cortical layers and lateral septum. Few data exist on MR expression in the human brain and to our knowledge includes only three reports on differences between depressed patients and healthy controls (Lopez et al., 1998; Xing et al., 2004; Wang et al., 2008). These studies were focused on one or two brain regions, while no distinction was made between *MR* promoter splice variants. At least two human *MR* promoter splice variants exist, MRα and MRβ (Zennaro et al., 1995), which are differentially regulated by hormones (Zennaro et al., 1996) and show tissue-specific expression patterns (Zennaro et al., 1997). Animal studies show that the relative expression of the MR splice variants changes during development (Vazquez et al., 1998) and that these variants may be of relevance when dealing with challenges (Kang et al., 2009).

We hypothesize that MR mRNA levels are lower in the limbic brain of MDD patients. Relative mRNA levels of total MR, MRα, MRβ and total GR were assessed in 5 brain regions and comparisons were made between MDD patients and non-depressed subjects. We show that MRa and MRb were detectable throughout the limbic brain, while lower MR and GR mRNA levels were found in several brain regions of the MDD patients.

Methods

Subjects and selected brain regions

Human *postmortem* brain tissue of in total 12 subjects was obtained from the Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam, The Netherlands), which receives *postmortem* brain material following permission from the patient or a close relative for a brain autopsy and for the use of the brain material and clinical information for research purposes. Brains were dissected, snap-frozen in liquid nitrogen and stored at -80ºC. Tissue was obtained from 5 brain regions, namely the hippocampus (Hi), amygdala (Amg), inferior frontal gyrus (IFG), cingulate gyrus (CgG) and nucleus accumbens (Acb). The subjects included 6 patients that were clinically diagnosed with major depressive disorder (MDD) during their life and 6 non-depressed controls with neither a diagnosis of a CNS disease nor a history of psychotropic medication use. The controls were selected in order to match for sex, age, brain weight, postmortem delay and pH of the CSF (a measure for agonal state; $p > .31$ for all). **Table 1** gives an overview on the subjects' clinicopathological characteristics. The patients' diagnoses for MDD were verified by a certified psychiatrist (Dr. G. Meynen) retrospectively, using the subjects' medical record (including details on clinical features, visits to clinicians, family history and medication use) and the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV; American Psychiatric Association). The

medical records did not reveal any alcohol or other drug abuse by any of the 12 subjects. Only one of the MDD patients (07-033) was diagnosed with a comorbid disorder, namely a compulsory-obsessive personality disturbance. Nucleus accumbens was available from only three of the non-depressed subjects (NBB 01-045, 01-069 and 01-079) and four of the depressed subjects (NBB 97-057, 01-074, 06-011, and 07-033). Cingulate gyrus tissue was not available for one of the depressed subjects (NBB 97-057) and also inferior frontal gyrus tissue was not available for one of the depressed subjects (NBB 06-026) and medial frontal gyrus tissue was used instead.

Table 1 Clinicopathological data of the patients with major depressive disorder (marked grey) and the non-depressed controls (unmarked, this table was previously presented in Alt et al., 2010).

	NBB number	Sex	Age (yrs)	Braak stage	Post-mortem delay (h:min)	pH CSF	Brain weight (g)	Clock time at death	Medication use in the past	Medication use in the last 3 months	Cause of death
MDD1	07-033	M	88	2	6:37	6.26	1225	21:15	TCA	TCA	Multiple epileptic seizures
MDD2	06-011	F	60	1	4:20	ND	1080	16:10	SSRI, BZD Hal, Mo Tamoxifen	Hal	Legal euthanasia
MDD3	02-051	M	81	3	6:00	6.50	1345	15:30	TCA, Hal	Hal	Renal insufficiency
MDD4	01-074	M	45	0	7:00	6.55	1427	2:30	SSRI, BZD	SSRI	Brain haemorrhage
MDD5	06-026	M	70	1	7:15	6.50	1415	8:00	TCA, Hal	TCA	Respiratory insufficiency
MDD6	97-057	F	81	3	5:25	6.74	1313	9:05	Hal	Hal	Pneumonia/ dehydration
Mean (SD)	-	-	70.8 (16.0)	1.7 (1.2)	6:06 (1:05)	6.51 (0.17)	1300.8 (130.8)	12:05 (6:46)	-	-	-
HC1	01-005	F	77	1	19:45	6.21	1149	0:15	None	Mo	Malignant lymphoma
HC2	01-045	M	83	1	4:35	6.49	1422	19:05	None	Mo, BZD	Heart attack
HC3	01-069	F	68	1	5:45	6.97	1153	12:15	Tamoxifen, BZD	None	Legal euthanasia
HC4	01-079	F	90	3	4:45	6.50	1120	18:45	Digoxin	Digoxin	Heart failure after CVA
HC5	02-008	M	62	0	9:35	6.58	1175	10:00	None	None	Metastasized adenocarcinoma
HC6	05-034	M	56	0	14:00	7.03	1323	0:01	None	None	ND
Mean (SD)	-	-	72.7 (13.0)	1.0 (1.1)	9:44 (6:05)	6.63 (0.31)	1223.7 (120.7)	10:03 (8:28)	-	-	-

Notes: The two groups did not differ in sex (p= .56; χ^2-test), age (p= .87), brain weight (p= .34), postmortem delay (p= .52) and pH (p= .71) of the CSF (a measure for agonal state; Mann-Whitney U-test).

Abbreviations: BZD, benzodiazepine; COPD, compulsory-obsessive personality disturbance; CSF, cerebrospinal fluid; F, female; Hal, haloperidol; HC, healthy controls; MDD, major depressive disorder; Mo, morphine; M, male; NBB, Netherlands Brain Bank; ND, no data; none, no medication; SD, standard deviation; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

Braak stages, neuropathological distribution of neurofibrillary Alzheimer changes over the brain; stage 0, no neurofibrillary changes; stages I/II, mild/severe alterations in the entorhinal cortex; stage III, first involvement of the hippocampus. Clinically stages 0–II are unaffected controls, and in stage III mild cognitive impairment may start.

RNA isolation

Cryostat sections of 20 μm were cut on a Leica CM1900 cryostat (Leica Microsystems, Rijswijk, The Netherlands) and put in 1 ml of QIAzol Lysis Reagent (Qiagen, Hilden, Germany). Total RNA was isolated from 30 sections using the RNeasy Lipid Tissue kit (Qiagen) following the manufacturer's instructions. RNA was quantified on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and RNA quality was determined on a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number (RIN) was used as an indication for RNA quality (scale 1–10, with 1 being the lowest and 10 being the highest RNA quality). RNA samples were stored at -80°C until further analysis. For each tissue sample 1 out of 10 sections was stained with 0.1% cresylviolet and used for histological confirmation (for the resulting figures see (Alt et al., 2010)).

cDNA synthesis

Synthesis of cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad, Hemel Hempstead, UK). For each sample cDNA was synthesized based on an equal quantity of RNA (1 μg) and in a total volume of 50 μl at 42°C for 30 min. For each brain region, standards and controls without reverse transcriptase (-RT) were prepared from pooled samples. A -RT sample and a sterile PCR grade water sample were taken along during cDNA synthesis to control for contamination with genomic DNA. To control for variability in cDNA amplification efficiency, duplicate cDNA samples were synthesized in separate runs and qPCR results compared. After cDNA synthesis the samples were stored at -20°C until further analysis.

Primer design

Specific intron-spanning primers for total MR (exon 2–3; NCBI ID: NM_000901; forward: 5'–CTG AGT TCC TTT CCT CCT GTC–3' and reverse: 5'–GCC ACA GGT GAC TAC CCC AT–3'; 225 bp amplicon; annealing temp. 61°C), MRα (NCBI ID: NM_000901; forward: 5'–CAG GTA GAC GGC GAG AGA–3' and reverse: 5'–CCT GAG AAA CTT GAC CCC ACC–3'; 112 bp amplicon, annealing temp. 62°C), MRβ (NCBI ID: X97925 (genomic DNA sequence); forward: 5'–TCG CCG CCT CTT GTA GGG TA–3' and reverse: 5'–ACC CCA CCG TCT TTC CAT ATC–3'; 263 bp; annealing temp. 62°C), total GR (NCBI ID: NM_001024094; forward: 5'–TCT GAA CTT CCC TGG TCG AA–3' and reverse: 5'–GTG GTC CTG TTG TTG CTG TT–3'; 110 bp amplicon; annealing temp. 62°C; primers are different from the previous study (Alt et al., 2010)) and the three reference genes tubulin (*TUBB2A*; NCBI ID: NM_001069; forward: 5'–CTG GCA CCA TGG ACT CTG–3' and reverse: 5'–TCG GCT CCC TCT GTG TAG–3'; 124 bp amplicon; annealing temp. 61°C), β-actin (*ACTB*; NCBI ID: NM_001101; forward: 5'–GGC CAC GGC TGC TTC–3' and reverse: 5'–GTT GGC GTA CAG GTC TTT GC–3'; 208 bp amplicon, annealing temp. 62°C) and GAPDH (NCBI ID: NM_002046; forward: 5'–ATC ATC AGC AAT GCC TCC TGC–3' and reverse: 5'–ATG GCA TGG ACT GTG GTC ATG–3'; 107 bp amplicon, annealing temp. 62°C) were designed based on the corresponding mRNA sequences using Vector NTi (Invitrogen, Paisly UK). All primers were synthesized by Isogen Life Science (Maarssen, The Netherlands). Specificity of each primer pair was verified by standard PCR followed by sequencing analysis.

Quantitative PCR

Q-PCR reactions had a total volume of 10 μL containing 2 μL of LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.75 μl of each primer (10 μM) and 2.5 μL of template cDNA. The qPCR was performed on a LightCycler 2.0 (Roche Diagnostics) and the program included a 10 min pre-incubation at 95°C, followed by 45 cycles of 10 sec at 95°C, 10 sec at the required annealing temperature and 10 sec at 72°C. Specificity of amplification was verified by melting curve analysis and electrophoresis of part of the qPCR products on a 2% agarose gel. Each run included a sample with sterile PCR grade water, a -RT sample (see the section on cDNA synthesis) to control for contamination with genomic DNA and the commercial human hippocampal sample to control for variability between qPCR runs. For each target gene a standard curve was included consisting of six two-fold dilutions of cDNA (see the section on cDNA synthesis). Q-

PCR efficiencies (*E*) were high; *E*= 1.92–2.15 for the target transcripts, *E*= 1.86–2.13 for tubulin and *E*= 1.89–1.96 for GAPDH; except for β-actin *E*= 1.59–1.92.

Normalization and statistical analysis

Each sample of the first set of cDNA preparations was measured in duplicate (except for GAPDH) and the mean value of the duplicate measurements was used for further calculations. For each tissue sample two cDNA samples were synthesized in separate runs and the qPCR results based on the first set of cDNA samples were validated with the results based on the second set of cDNA samples, which were measured once. The relative number of copies of each target transcript was calculated by $10^{10} \times E^{-ct}$ ($E= 10^{-(1/slope)}$) (Kamphuis et al., 2001). For normalization, the number of copies of the target gene was divided by the number of copies of tubulin, as tubulin showed to be the most stable reference gene. Normalization with β-actin or GAPDH resulted in similar results on differential expression between brain regions and between groups. Differences between the depressed and non-depressed groups were determined by a nonparametric Mann-Whitney *U*-test, as the assumption of a normal distribution was not met. Statistical analysis was conducted with SPSS (version 16.0 for Mac OS X; SPSS Inc., Chicago, IL, USA). A two-sided *p*-value below .05 was considered statistically significant.

Results

RNA quality

The RIN values did not differ significantly between the MDD patients (mean ± SD; 7.2 ± 0.7) and controls (7.3 ± 0.8; t(52)= 0.33, *p*= .74, independent samples *t*-test) or between the different brain areas (Hi 7.1 ± 0.9; Amg 7.1 ± 0.7; IFG 7.6 ± 0.6; CgG 7.1 v 0.6; Acb 7.6 ± 0.9; *p*= .23, Kruskal Wallis test).

Relative expression of total MR and total GR mRNA in non-depressed brain

Levels of total MR mRNA as well as total GR mRNA were determined in 5 brain regions from 6 non-depressed subjects and calculated relative to the total MR levels in the hippocampus (**Figure 1**). Total MR and GR could be detected in all brain regions. The highest levels were found in the hippocampus, while in the amygdala, inferior frontal gyrus, cingulate gyrus and nucleus accumbens expression levels were around 20 to almost 100 times lower for the MR and 10 to 20 times lower for the GR. The results show that MR and GR expression patterns in the 5 brain regions were similar, while the hippocampus was the only region with a MR/GR ratio above 1 (total MR levels 1.5 times higher than total GR). In the other four regions the MR/GR ratio was below 1 (total MR levels respectively 2.7; 2.7; 1.3 and 1.6 times lower than GR).

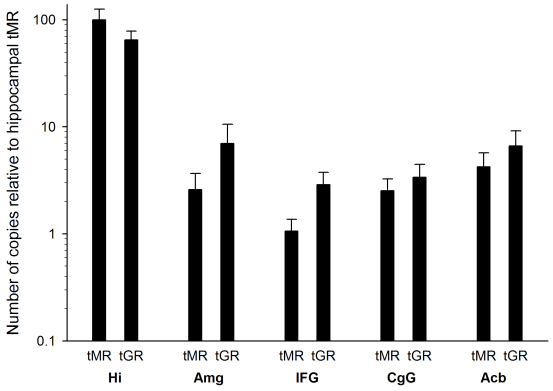

Figure 1 Mean relative number of copies of total MR (tMR) and total GR (tGR) transcript compared between 5 brain regions of 6 non-depressed subjects. The number of copies of total MR and GR mRNA within each brain region was calculated relative to total MR mRNA expression detected in the hippocampus (set at 100%). The figure presents data after normalization with tubulin. Note that expression levels are plotted on a log scale.

Relative expression of total MR mRNA and its splice variants in non-depressed brain

The relative levels of total MR, MRα and MRβ mRNA were compared within 5 brain regions from 6 non-depressed subjects. **Figure 2** shows that both MRα and MRβ could be detected in all brain regions. MRα expression levels constituted 18–67% of total MR, while MRβ expression levels were very low, representing 0.3–2.5% of total MR; MRα levels were around 10 to 60 times higher than MRβ in the different regions. The sum of MRα and MRβ expression did however not add up to the total MR levels.

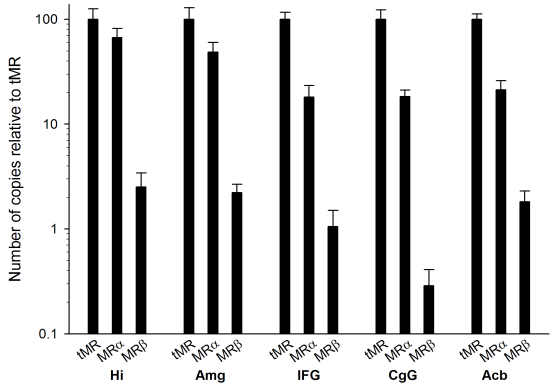

Figure 2 Mean relative number of copies of total MR (tMR), MRα and MRβ within 5 brain regions of 6 non-depressed subjects. For each brain region the number of copies of MRa and MRb is expressed relative to total MR mRNA expression (set at 100%). The figure presents data after normalization with tubulin. Note that expression levels are plotted on a log scale.

One significant outlier was found for MRβ mRNA expression in the inferior frontal gyrus in the non-depressed group. Excluding this subject (NBB 01-069) weakened the difference between the depressed and non-depressed group but it was still significant (p= .02). MR or GR expression levels did not differ between the males and females, although MR and GR expression seemed somewhat higher in amygdala and cingulate gyrus of the female subjects.

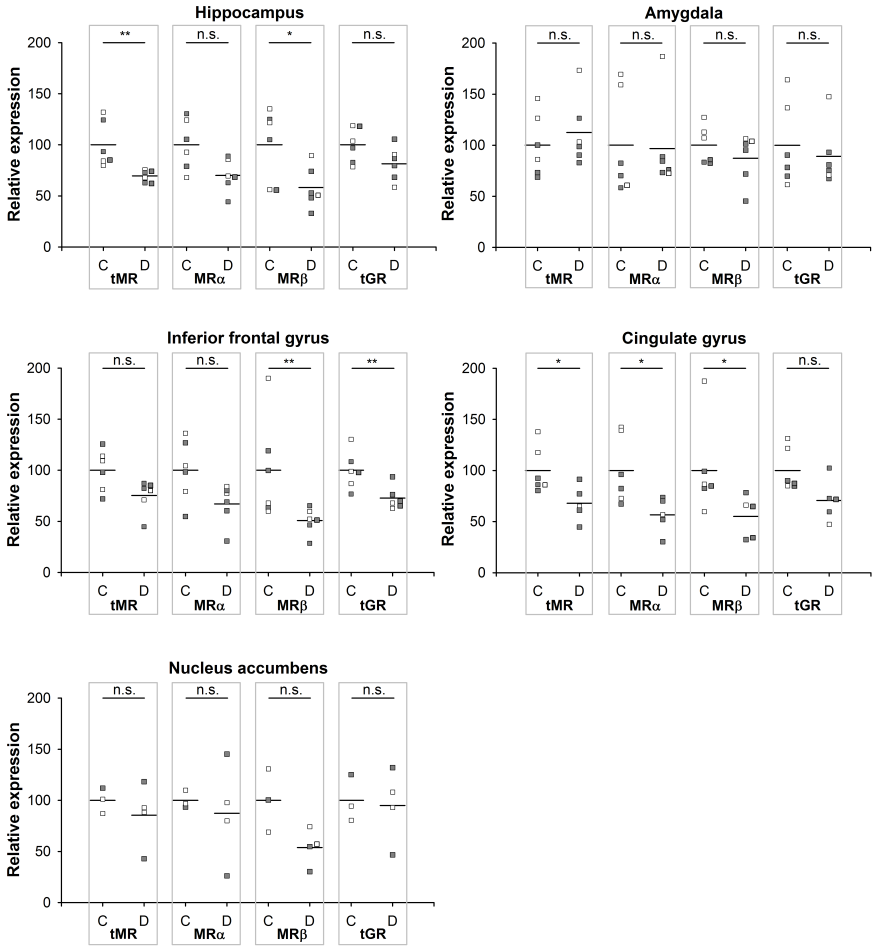

Figure 3 Mean expression of total MR (tMR), MRα, MRβ or total GR (tGR) mRNA levels in 5 brain regions of MDD patients (D) relative to non-depressed subjects (C). Male subjects are indicated with grey squares, female subjects are indicated with open squares. For the nucleus accumbens only three (C) or four (D) tissue samples were available and for the cingulate gyrus only five tissue samples were available for the MDD (D) group. Excluding the one significant outlier with high MRβ mRNA expression in the inferior frontal gyrus (control NBB 01-069) weakened the difference between the two groups but it was still significant (p< .05). The figure presents data after normalization with tubulin. Note that the expression levels are plotted on a linear scale. * p< .05; ** p< .01; n.s.= not significant

MR/GR ratio in brain of depressed compared to non-depressed subjects
For the 6 depressed subjects the total MR/GR ratio was calculated in 5 brain regions and compared with the 6 non-depressed subjects. Patterns of expression of total MR relative to total GR between the different brain regions were comparable; although there were some significant differences in MR and GR transcript levels between both groups, this did not significantly change the MR/GR ratio.

Discussion

The present study shows the highest MR mRNA expression levels in the human hippocampus. In addition, relative to total GR, total MR expression was higher in the hippocampus and lower in the cingulate gyrus, inferior frontal gyrus, amygdala and nucleus accumbens. Both MR splice variants, MRα and MRβ, were detected in all 5 brain regions, although expression levels of MRβ were around 10 to 60 times lower than for MRα over the various brain regions. Comparing brain tissue from MDD patients to brain tissue from non-depressed subjects showed significantly lower MR levels in the hippocampus, inferior frontal gyrus and cingulate gyrus. The differences were region and transcript specific. However, no significant difference in MR/GR ratio was found.

Our data on total MR distribution are comparable to earlier studies showing in various species higher corticosteroid receptor expression levels in the hippocampus compared to other parts of the brain (de Kloet and Reul, 1987; Meyer et al., 1998; Sanchez et al., 2000; Pryce et al., 2005). Moreover, higher hippocampal MR mRNA levels relative to GR mRNA have previously been reported for rats, tree shrews and monkeys (Reul and de Kloet, 1985; Bohn et al., 1994; Meyer et al., 1998; Patel et al., 2000; Sanchez et al., 2000; Pryce et al., 2005). In addition, comparable with non-human primate brain MR expression was around 2 to 3 times lower than for GR in the amygdala or prefrontal cortex (PFC) (Patel et al., 2000; Sanchez et al., 2000; Pryce et al., 2005). However, we did not make subregional distinctions, whereas the aforementioned reports show that MR vs. GR expression levels are strongly subregion dependent (for a review on central MR and GR expression patterns see (Pryce, 2008)).

The relative expression levels of the two MR splice variants showed similarities with rat studies. The MRα versus MRβ ratio in the rat hippocampus varies throughout development (Vazquez et al., 1998), with MRβ being predominantly expressed during the second week after birth and showing a subsequent decrease up into the adult age. At adult age MRβ expression seems to be lower than MRα expression. In the present study MRβ expression levels were much lower compared to MRα. As all 12 subjects were of middle age or older (mean age 71.8 ± 13.9) this may suggest that central MRβ expression decreases even further during aging. The levels of expression of MRα together with MRβ did not add up to that of total MR. This discrepancy was also found earlier (Kwak et al., 1993; Zennaro et al., 1997; Vazquez et al., 1998) and suggests that there are additional unidentified MR transcripts. Indeed, in rats a third *MR* alternative first exon has been identified, exon1g

(Kwak et al., 1993) and for the human *GR* at least 9 promoter splice variants exist (Turner et al., 2006; Presul et al., 2007). However, it is important to note that results obtained with different probes or primer sets should be interpreted and compared with caution as they can differ in signal intensity or efficiency.

In brain tissue of the depressed subjects significantly lower MR transcripts levels (30-50%) were detected in the hippocampus, cingulate gyrus and inferior frontal gyrus tissue, while total GR expression was lower (30%, significant or trend) in the cingulate gyrus and inferior frontal gyrus. No difference in receptor expression was found in the amygdala or nucleus accumbens. A trend for lower MR levels in the hippocampus has previously been reported for depressed subjects who committed suicide (Lopez et al., 1998), while no difference in GR expression was detected. A significantly lower or trend in lower MR or GR expression was also found in the PFC of patients suffering from bipolar disorder or major depression (Webster et al., 2002; Xing et al., 2004). On the other hand, in laser microdissected paraventricular tissue of the hypothalamus of patients diagnosed with depression, MR mRNA expression was found increased along with enhanced expression of CRH, as is commonly found in depressed patients (Wang et al., 2008). This indicates that MR regulation may differ between various brain areas, but the mechanism behind this different regulation is still unclear. The results seem in line with the idea that both the decreased MR expression in the hippocampus and the increased MR expression in the hypothalamus are characteristic for the HPA hyperactivity that is often observed in depressed patients. In the previous study measuring GR expression in the same samples, no significant group difference in total GR expression was reported for any of the 5 studied brain regions (Alt et al., 2010), although expression levels were somewhat decreased in the MDD patients (Alt, personal communication). This difference may be due to the use of different primers sets and techniques. In the current study it is interesting to note that, despite its low expression, MRβ was significantly decreased in three out of five brain regions studied. With respect to the MR/GR ratio no significant group differences were found.

Disturbed central receptor expression can be due to both genetic and/or experience-related factors and present a risk factor for depression. In addition, environmental inputs triggering stress reactions can modulate corticosteroid receptor expression either directly or as part of endocrine and behavioral adaptations (Holsboer, 2000; de Kloet et al., 2005; Oitzl et al., 2010). For the *MR* gene we identified multiple common single nucleotide polymorphisms (SNPs) and haplotypes modulating *MR* transcription, protein expression and/or transactivation in cell lines (DeRijk et al., 2006; van Leeuwen et al., 2010a, 2010b, 2011) Chapter 3). Possibly, these functional genetic variants relate to inter-individual variability in MR expression in the brain. However, the current number of subjects was too small to appropriately test this. We were able to show that these SNPs relate to differences in physiological and psychological coping systems, potentially predicting the risk of psychopathology (DeRijk et al., 2006; Kuningas et al., 2007; van Leeuwen et al., 2010a, 2011) (Chapter 4-6). Variability in corticosteroid receptor expression may result from stress during life or just before death (Gesing et al., 2001; Lai et al., 2009). Differences in receptor expression might also result from epigenetic variability, such as changes in methylation of

the genomic DNA due to environmental influences during development (Weaver et al., 2004).

As hippocampal MR is important for HPA reactivity, inappropriate MR signaling in this brain structure may lead to significant HPA disturbances. MDD patients often show HPA hyperactivity, which can be normalized by long-term treatment with antidepressants (Holsboer, 2001). Various antidepressant compounds are known to upregulate hippocampal MR (or GR) expression, preceding clinical relief (Seckl and Fink, 1992; Barden et al., 1995; Lopez et al., 1998; Bjartmar et al., 2000; Zobel et al., 2004). Aberrant MR signaling in the hippocampus, inferior frontal gyrus and cingulate gyrus could underlie the behavioral and cognitive problems of MDD patients. Hippocampal MR is thought to be involved in appraisal and behavioral response selection to novel situations (Oitzl et al., 1994), while the inferior frontal gyrus and cingulate gyrus are known to be implicated in these functions (Phillips et al., 2003; Zhang et al., 2004). Furthermore, inappropriate functioning of these regions may induce emotional problems like anxiety, which is modulated in mouse mutants carrying forebrain specific changes in MR expression (Brinks et al., 2007b; Rozeboom et al., 2007). The specific functions of MRα and MRβ are as yet not fully known. The present study shows that MRβ levels were low compared to MRα, which may suggest a limited functional role of MRβ in the adult or aged brain. However, recent *in vitro* and *in vivo* studies showed that specifically MRβ is upregulated in neurons in response to specific stressors, mediating neuronal survival (Macleod et al., 2003; Kang et al., 2009).

The present results are limited by the size and heterogeneity of the study groups. An important potential confounder was the use of antidepressants by the MDD patients, which, as already mentioned, potentially upregulate central MR. Nevertheless, despite the use of antidepressant drugs central MR mRNA levels were significantly lower relative to the levels observed in the non-depressed subjects. Variability in MR expression may also result from stress during life or just before death (Gesing et al., 2001; Lai et al., 2009). Moreover, differences in MR expression may be due to the old age of the subjects, as animal studies indicate that MR expression decreases with aging (van Eekelen et al., 1991). However, the mean age of the two study groups was similar. Furthermore, differences in mRNA expression do not necessarily correlate with similar differences in protein expression and the differences may be subregion specific.

The present data are indicative for aberrant MR expression in MDD patients, which possibly is linked to the disturbances in the HPA axis, mood and cognitive performance often observed in MDD patients. It is the first study that shows relative expression levels of the two known human *MR* promoter splice variants, which potentially confer differential MR regulation and functioning in the brains of healthy and depressed subjects. Future studies should include a higher number of subjects of both sexes, preferentially with a known history of stressful life events.

Supplemental data

Although the number of subjects is too small to test for differences in frequencies of the *MR* -2G/C and I180V genotypes and their respective haplotypes between the depressed patients and non-depressed controls, we did assess the subjects' genotypes and diplotypes (**Supplemental Table 1**).

Supplemental Table 1 Frequencies of the *MR* -2 G/C and I180V genotypes and their respective haplotypes among the depressed patients and non-depressed controls

MR gene variant	Non-depressed controls	Depressed patients
-2 G/G	3	3
-2 G/C	0	0
-2 C/C	3	3
180 I/I (A/A)	4	4
180I/V (A/G)	2	1
180 V/V (G/G)	0	1
Hap 1 -2G/180A	5	6
Hap 2 -2C/180A	5	3
Hap 3 -2C/180G	1	3
Hap 4 -2G/180G	1	0

Of note is that, compared to the controls, the haplotype 2 frequency is lower and the haplotype 1+3 frequency is higher in the depressed subjects. In addition, the subjects with the highest total MR expression (both controls) in the hippocampus were -2 C/C homozygotes and 180 A/G heterozygotes and carried one or two haplotype 2 alleles.

Acknowledgements
This work was supported by the International Research Training Group (IRTG) funded by the DFG (GRH 1389/1) and NWO (DN95-420), psychiatric hospital Rivierduinen, the Kassenaar Fonds and the Royal Netherlands Academy for Arts and Sciences.