Mineralocorticoid receptor in human brain : a key player in resilience

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Chapter 8

General discussion

Outline

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The objective of this thesis research was to extend and to validate the knowledge on the mineralocorticoid receptor (*MR*) gene variability and to examine its implications for human mental health. We have identified three common *MR* gene haplotypes of which haplotype 2 (freq. ~0.38) contains SNPs in the *MR* gene promoter region that enhance the synthesis of MR when compared to haplotypes 1 and 3. We found that this haplotype 2 associates with resilience and protects against depression in women and not in men. We therefore conclude that the *MR* gene modulates susceptibility to stress and depression.

The above conclusion is based on the *MR* in the limbic brain, which not only has a high affinity for the mineralocorticoid aldosterone, but also for the naturally occurring glucocorticoids cortisol (humans) and corticosterone (rodents), while also progesterone and deoxycorticosterone can bind. Hence, the MR is a promiscuous receptor, which in the human brain *sees* predominantly cortisol because of its much higher concentration than aldosterone. In epithelial cells such as kidney the MR selectively mediates aldosterone effects on electrolyte balance, volume regulation and blood pressure (Funder, 2005). This aldosterone selectivity is due to the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 (Seckl, 1997), which converts cortisol to its bio-inactive metabolite cortisone. 11β-HSD type 2 is absent in non-epithelial tissues like the vessels, heart and brain, explaining why in these tissues the MR functions as a high affinity cortisol receptor. In the brain the 11β-HSD type 1 isoform is present which generates bio-active cortisol.

In the limbic brain the MR mediates cortisol effects on stress-induced neuronal excitability as well as on emotional arousal and behavioral adaptation. The MR mediates cortisol effects in a complementary fashion with the lower affinity glucocorticoid receptor (GR). Ample lines of evidence suggest that an imbalance in central MR vs. GR mediated effects may result in disturbances in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis and its end product cortisol, altered emotional arousal and impaired cognitive performance, with potential implications for psychopathology. At the start of this PhD research our group had identified two common *MR* single nucleotide polymorphisms (SNPs; -2G/C and I180V) that affect MR protein expression and activity in cells and that associate with variability in neuroendocrine regulation in healthy individuals.

8.1 Main findings

Molecular studies

MR expression in limbic brain

It has been proposed that stress-related disorders like anxiety and depression involve an imbalance in MR vs. GR mediated actions in the brain (de Kloet et al., 1998; Holsboer, 2000). In order to test this hypothesis, a primary goal is to assess brain MR and GR expression. However, only a few reports exist on comparisons of MR expression between the *postmortem* brains of patients that had suffered from major depressive disorder (MDD) and of non-depressed subjects (Lopez et al., 1998; Xing et al., 2004; Wang et al., 2008). We have assessed MR mRNA expression in several limbic brain structures using *postmortem*

brain tissue from six non-depressed subjects and six MDD patients **(Chapter 2)**. MR was expressed in all brain regions that were analyzed, with the highest expression levels in the hippocampus and much lower expression levels (i.e. 20 - 100 times lower) in the amygdala, cingulate gyrus, inferior frontal gyrus and nucleus accumbens. We discovered that the central MR expression was approximately 30% lower in the depressed patients, particularly in the hippocampus, inferior frontal gyrus and cingulate gyrus. Also GR expression was slightly but significantly decreased in the depressed brain. Together the changes in MR and GR expression did not result in a change of the MR/GR mRNA ratio.

It is known that MR expression is induced by corticotrophin releasing hormone (CRH) released during acute psychological stress (Gesing et al., 2001), but chronic stress is known to suppress MR. Importantly, although the patients were using antidepressants, which are known to induce MR and GR expression (Seckl and Fink, 1992; Lopez et al., 1998; Bjartmar et al., 2000), still MR expression was significantly lower in the brains of the depressed patients. This suggests that central MR expression may be even lower among drug naïve MDD patients. An additional interesting finding was that the MRβ splice variant was significantly less expressed in the hippocampus, inferior frontal gyrus and cingulate gyrus of the depressed subjects as compared to the control brains. As MRβ is known to be important for neuronal survival at least in response to physiological stress, like hypothermia and anoxia (Kang et al., 2009), a decrease in MRβ expression may affect the maintenance of neuronal integrity (Joëls et al. 2008). This needs however further investigation.

Postmortem, depressed patients had lower MR mRNA expression in their limbic brain than non-depressed controls, despite their use of antidepressants.

***In vitro* functionality of 5' *MR* haplotypes**

Our approach is to perform genetic association studies with SNPs for which we have indications that they affect gene activity. SNPs that affect gene functioning *in vitro* potentially are also active *in vivo*, strengthening the reliability of associations found between the candidate gene and specific phenotypes. Associations found with SNPs for which it is not known that they are functional possibly mask real 'risk' alleles. SNPs in a gene's promoter may affect gene transcription and eventually protein expression, for example by interfering with transcription factor binding. Indeed, multiple SNPs in the *MR* gene promoter region were found to modulate promoter activity, with a specific combination or haplotype (haplotype 2, freq. ~0.38) resulting in higher gene transcription compared to the other two common haplotypes 1 and 3 (**Chapter 3**). The effect on gene transcription synergizes with the effects previously found with the SNPs located in exon 2. The promoter haplotype 2 SNPs are linked to the exon 2 variants and jointly this combination of SNPs results in the highest gene translation and transactivational capacity (van Leeuwen et al., 2011). Together

this indicates that haplotype 2 results in the highest MR mRNA and protein levels and the highest transactivation of its target genes. However, this inference should be verified by testing the different *MR* promoters (containing the combination of SNPs designated as haplotype 1, 2, or 3) combined with the different variants of exon 2 (also haplotype 1, 2, or 3).

The present results underline that in genetic research the naturally occurring combinations of SNPs can have quite different effects as compared to the isolated single SNPs. In our case, the functional effect of a specific combination of SNPs, haplotype 2, was distinct from the others, haplotype 1 and 3. Of note is that caution should be exercised when extrapolating the *in vitro* findings to the *in vivo* situation, since the effects of SNPs may vary depending on the context. Nevertheless, we showed that the *MR* SNPs can modulate MR molecular activity and are not just 'silent' SNPs.

A common (freq. ~0.38) gene variant at the 5' end of the *MR* gene, haplotype 2, results in higher transcription and translation of *MR* and causes enhanced transactivation of its target genes.

Endophenotypes

Endophenotypes are proposed to be biologically less complex than the actual psychiatric mental disorders and are therefore thought to be more suitable for the identification of biologically more homogeneous groups of subjects, also at the level of genetics (Gottesman and Gould, 2003). Here we focused on psychological traits and HPA axis reactivity.

The effects of 5' *MR* haplotypes on personality in subjects selected from the general population

In this thesis two distinct but complementary association studies of the common functional 5' *MR* haplotypes are described for traits known to influence coping behavior and risk of depression. In a first study among elderly subjects an association was found between *MR* haplotype 2 (the one that results in the highest molecular MR activity *in vitro*) and heightened dispositional optimism, a trait reflecting the individual's generalized favorable expectancies for the future (**Chapter 4**). In a second study among students an association was found between the same *MR* haplotype 2 and less cognitive reactivity to sad mood (**Chapter 5**). Specifically, *MR* haplotype 2 was associated with fewer thoughts of hopelessness and in addition with less rumination. Intriguingly, in both studies effects were restricted to women. In other words, female subjects carrying *MR* haplotype 2 have a lower risk of depression, seemingly in part through their personality and coping behavior. Importantly, the strength of these two studies combined is that the results show a similar

association in opposite directions, that is, *MR* haplotype 2 was related to higher scores for a 'positive trait' and with lower scores for 'negative traits'.

5' *MR* haplotype 2 associates with a lower psychological risk of depression, but only in women.

5' *MR* haplotypes and their effects on cortisol levels in depressed patients

Depression is often characterized by HPA disturbances. As the *MR* plays an important role in the regulation of diurnal HPA activity, effects of the functional *MR* gene variants may be expected on diurnal cortisol levels. We have previously found effects of *MR* SNPs on non-stress and stress-related cortisol secretion in healthy individuals (DeRijk et al., 2006; Kuningas et al., 2007; van Leeuwen et al., 2010a; van Leeuwen et al., 2011). Here we tested for effects on circulating cortisol in a large group of MDD patients that participate in the Netherlands Study of Depression and Anxiety (NESDA, **Chapter 6**). Actually, in the total group of patients only a small effect of the *MR* -2G/C SNP on the cortisol awakening response (CAR) was found in the depressed women. Importantly, when the depressed subjects were split for the frequent use of selective serotonin reuptake inhibitors (SSRIs) the -2 C-allele was found to associate with an attenuated CAR compared to the G/G genotype in men and women using SSRIs and not in the patients that were not using SSRIs. No significant effect was found for the *MR* I80V SNP. At the haplotype level, when using SSRIs haplotype 2 was significantly associated with a decrease in morning cortisol, as compared to haplotype 1. Also haplotype 3 was associated with a lower CAR, but this effect was smaller.

5' *MR* gene variability modulates morning cortisol levels in MDD patients using SSRIs.

Recently, based on the NESDA data the CAR was found to associate with symptom severity according to an inverted U-shape; both very high and low symptom severity was associated with a low CAR (Wardenaar et al., 2011). Moreover, a low CAR associates with a more chronic course of the disease (Vreeburg, 2010b). We showed that clearly the SSRIs interact with the patient's *MR* genotype in their effect on the CAR (see also 'Interaction with antidepressants'). Some of the SSRI users had a heightened and prolonged CAR, while others had a flattened CAR. Thinking of the results reported by Wardenaar *et al.* (Wardenaar et al., 2011) and Vreeburg *et al.* (Vreeburg, 2010b) one might question whether this is desirable. Possibly antidepressants establish their clinical effect through the MR as

they are known to induce central MR (and GR) expression (Seckl and Fink, 1992; Bjartmar et al., 2000). Therefore, it would be important to know whether the SSRI-by-*MR* haplotype interaction is also associated with differences in clinical response. Longitudinal studies on clinical response are warranted.

Psychopathology

The effects of 5' *MR* haplotypes on the risk of major depressive disorder

Building further on the results of our neuroendocrine and psychological studies, the follow-up test should be to determine whether there is an association between the functional *MR* gene variants and the diagnosis of depression. The students that participated in the study aimed to examine their level of cognitive reactivity to sad mood also answered the question if they had ever been diagnosed with depression (**Chapter 5**). Among those students an association was found with *MR* haplotype 2, that is, the risk of self-reported diagnosis of depression did decrease with the number of *MR* haplotypes 2. Again, only in women haplotype 2 was associated with a lower risk of depression. In order to verify those results, data were used from a large genome-wide association study (GAIN-MDD), which was based on two well-established Dutch cohorts, the NESDA cohort and the Netherlands Twin Registry (NTR; **Chapter 7**). Only in women a trend towards a relation between *MR* haplotype 2 and a lower risk of depression was found. However, as these sex-differences may be due to sex steroids, the data were additionally verified for an interaction with the mean age for menopause (~51 yrs.). Strikingly, there was indeed an interaction effect; only in the premenopausal women an association was found between haplotype 2 and fewer diagnosis of depression, particularly in the women aged 41 years or younger.

5' *MR* haplotype 2 associates with a lower risk of depression, but only in premenopausal women.

Conclusion

The data show that the gene variants encoding altered MR molecular activity affect human physiological and psychological functioning (see **Table 1** for an overview). The data suggest that decreased MR activity linked to haplotype 1 and 3 enhances the risk of depression. Accordingly, MR expression was lower in several limbic brain regions of depressed patients. The *MR* gene variant that resulted in the highest molecular MR activity *in vitro*, haplotype 2, related to the highest resilience against depression *in vivo*. The strength of the combined data is that we were able to show a similar association repeatedly and in small as well as in large groups of subjects. We have found a genetic marker, which has a high frequency in the population (freq. ~0.38), that not only identifies individuals that are psychologically at a lower risk of depression, but also truly associates with fewer diagnosis of depression,

despite the fact that in this final case-control study we are dealing with a heterogeneous population.

8.2 Interaction effects

Previous data have shown that the *MR* gene variants significantly affect MR molecular activity and human physiological and psychological functioning, often in interaction with other factors. The present data clearly add to this notion.

Gender differences

The associations found between the *MR* haplotypes and the psychological traits and the risk of depression were restricted to women. Sex differences in brain functioning are becoming increasingly acknowledged (Jazin and Cahill, 2010) and differences in HPA activity between males and females are widely documented (Kudielka and Kirschbaum, 2005; Vreeburg et al., 2009b; van Leeuwen et al., 2010a). With respect to MR functioning this is important, as MR activity is in part depending on ligand availability. The *in vitro* studies performed by our group have shown that the effects of the exon 2 variants on MR transactivational capacity largely depend on the concentration of cortisol that was applied to the cells; transactivation did not differ between the *MR* gene variants at high or low cortisol concentrations (10^{-8} or 10^{-12} M) but differed significantly with intermediate concentrations around the EC50 and Kd of the receptors (10^{-9} to 10^{-11} M) that are thought to be in the physiological range (DeRijk et al., 2006; van Leeuwen et al., 2010a, 2011). A remote possibility is that the circulating cortisol levels of women rather than that of men fall within this range. Moreover, sex differences in HPA activity may be due to the effects of sex steroids on MR (and GR) expression and ligand binding. Estrogens and androgens are known to modulate MR mRNA and protein expression, while progesterone can also bind the MR and have agonistic or antagonistic effects (Carey et al., 1995; Castren et al., 1995; Turner, 1997; Quinkler et al., 2002). Possibly these sex steroid effects depend on the *MR* haplotypes, which extend into the regulatory promoter region of the *MR* gene. Additional *in vitro* studies could clarify this.

Sex-dependent associations between the 5' *MR* haplotypes and psychological wellbeing may be due to an interaction between the *MR* gene variants and sex steroids.

Table 1 Overview of 5' *MR* gene variants and functionality

Level	-2G/C C-allele (freq. ~0.50)	I180V V-allele (freq. ~0.12)	Hap 2 in exon 2 (-2C/180I; freq. ~0.40)	Promoter hap 2 (8 SNPs, linked to hap 2 in exon 2; freq. ~0.40)
Molecular	mRNA ~ (Van Leeuwen et al., 2010b)	-	-	**mRNA ↑ (highest) (Chapter 3)**
	Protein ↑ (Van Leeuwen et al., 2010b)	Protein ~ (DeRijk et al., 2006)	Protein ↑ (highest), ≈ hap 3 (Van Leeuwen et al., 2011)	-
	Ligand binding with cort Kd ~, Bmax ↑ (Van Leeuwen, Thesis)	Ligand binding with cort Kd ~, Bmax ~ (Van Leeuwen et al., 2011)	Ligand binding with cort Kd ~, Bmax ↑, ≈ hap 3 (Van Leeuwen, Thesis)	-
	Transactivation with cort, aldo, dex ↑ (Van Leeuwen, 2010a, 2010b, 2011)	Transactivation with cort ↓ with aldo ~ (DeRijk et al, 2006)	Transactivation with cort ↑ (highest), ≈ hap 3 (Van Leeuwen et al., 2011)	-
	Transactivation with aldo ↓ (Arai et al., 2003)	*Transactivation with aldo ↓ (Arai et al., 2003)*	-	-
Endophenotype	Morning cortisol in elderly M+F ↓ (Kuningas et al., 2007)	Morning cortisol in elderly M+F ~ (Kuningas et al., 2007)	-	
Physiological	Morning cortisol after dex M ↓/F ↑ (Van Leeuwen et al., 2010a)	Morning cortisol after dex M ↓/F ~ (Van Leeuwen et al., 2010a)	-	
	Morning ACTH after dex M/F ~ (Van Leeuwen et al., 2010a)	Morning ACTH after dex M/F ~ (Van Leeuwen et al., 2010a)	-	
	-	Cortisol response to stressor M ↑ (DeRijk et al., 2006)	Cortisol response to stressor M+F ↑ (Van Leeuwen et al., 2011)	
	-	ACTH response to stressor M ~ (DeRijk et al., 2006)	ACTH response to stressor M+F ↑ (Van Leeuwen et al., 2011)	
	-	Heart rate response to stressor M ↑ (DeRijk et al., 2006)	Heart rate response to stressor M+F ↑ (Van Leeuwen et al., 2011)	
	-	*Cortisol response to stressor M+F ~ (Ising et al., 2008)*	-	
	-	*ACTH response to stressor M+F ~ (Ising et al., 2008)*	-	
	-	*ACTH just before 2nd stressor M+F ↑ (Ising et al., 2008)*	-	
	Systolic blood pressure M ↓/F ~ (Van Leeuwen et al., 2011)	-	-	
	Diastolic blood pressure M/F ~ (Van Leeuwen et al., 2011)	-	-	
	Systolic/diastolic blood pressure M+F ~ (Tobin et al., 2008)	*Systolic/diastolic blood pressure M+F ~ (Tobin et al., 2008)*	-	
	Urinary sodium/potassium/calcium M+F ~ (Tobin et al., 2008)	*Urinary sodium/potassium/calcium M+F ~ (Tobin et al., 2008)*	-	
	-	*Hypertension M/F ↓ (Martinez et al., 2009)*	-	
Psychological	-	-	Perceived chonic stress at work M+F ↓ (Van Leeuwen et al., 2011)	
	Optimism in elderly M ~/F ↑ (Chapter 4)	**Optimism in elderly M/F ~ (Chapter 4)**	**Optimism in elderly M ~/F ↑ (Chapter 4)**	
	Cognitive reactivity to sad mood M ~/F ↓ (Chapter 5)	Cognitive reactivity to sad mood M/F ~ (Chapter 5)	Cognitive reactivity to sad mood M ~/F ↓ (Chapter 5)	
	Hopelessness M ~/F ↓	**Hopelessness M/F ~**	**Hopelessness M ~/F ↓**	
	Acceptance/coping M/F ~	Acceptance/coping M/F ~	Acceptance/coping M/F ~	
	Aggression M ~/F ↓	Aggression M/F ~	Aggression M ~/F ↓	
	Perfectionism/control M/F ~	Perfectionism/control M/F ~	Perfectionism/control M/F ~	
	Risk aversion M ~/F ↓	Risk aversion M/F ~	Risk aversion M ~/F ↓	
	Rumination M ~/F ↓	**Rumination M/F ~**	**Rumination M ~/F ↓**	
	Neuroticism M ~/F ↓ (Chapter 5)	Neuroticism M/F ~ (Chapter 5)	Neuroticism M ~/F ↓ (Chapter 5)	
	-	-	Neuroticism M/F ~ (DeRijk et al., 2011)	
	-	-	Neuroticism in depression/anxiety patients M/F ~ (Hap 3 M+F ↓) (DeRijk et al., 2011)	
	-	*Non-stress reward learning M/F ↑ (Bogdan et al., 2010)*	-	
	-	*Stress reward learning F ↓ (Bogdan et al., 2010)*	-	
Psychopathology	Feelings of depression M+F ~ (Kuningas et al., 2007)	Feelings of depression M+F ↑ (Kuningas et al., 2007)	-	
	(Symptoms of) depression and/or anxiety M+F ~ (Van Leeuwen, Thesis)	(Symptoms of) depression and/or anxiety M+F ~ (Van Leeuwen, Thesis)	(Symptoms of) depression and/or anxiety M+F ~ (Van Leeuwen, Thesis)	
	Symptoms of depression and/or anxiety M+F ↓ (Van Leeuwen, Thesis)	Symptoms of depression and/or anxiety M+F ~ (Van Leeuwen, Thesis)	Symptoms of depression and/or anxiety M+F ↓ (Van Leeuwen, Thesis)	
	Symptoms of anxiety M ↓/F ~ (Van Leeuwen, Thesis)	Symptoms of anxiety M ~/F ~ (Van Leeuwen, Thesis)	Symptoms of anxiety M/F ~ (Van Leeuwen, Thesis)	
	Symptoms of depression M/F ~ (Van Leeuwen, Thesis)	Symptoms of depression M/F ~ (Van Leeuwen, Thesis)	Symptoms of depression M/F ~ (Van Leeuwen, Thesis)	
	Self-reported diagnosis of depression M ~/F ↓ (Chapter 5)	**Self-reported diagnosis of depression M/F ~ (Chapter 5)**	**Self-reported diagnosis of depression M ~/F ↓ (Chapter 5)**	
	Diagnsosis of depression M ~/F (< 41 yrs.) ↓ (Chapter 7)	**Diagnsosis of depression M/F ~ (Chapter 7)**	**Diagnsosis of depression M ~/F (< 41 yrs.) ↓ (Chapter 7)**	
	-	*Symptoms of depression and/or anxiety M+F ~ (Bogdan et al., 2010)*	-	

Notes: Results of other research groups are shown in italic text. Important results of the present thesis are shown in bold text.

Still, a role for sex hormones may vary in relevance since effects of the *MR* gene variants were observed in women of all ages. The association between the *MR* haplotypes and cognitive reactivity to sad mood was found in young female students. Also the association with the risk of self-reported depression was found in these same young female students. In the GWA study the *MR* haplotype 2 associated with a lower risk of depression in women aged 41 years or younger without interference of oral contraceptives. On the other hand, the association with optimism was found in postmenopausal women, who have low circulating female sex hormones. However, the relationship between the *MR* gene variants and differences in optimism levels might be determined already at an early age, as it was described to be a relatively stable trait (Giltay et al., 2006b). Optimism is in part influenced by, for example, a person's socio-economic status (SES), particularly as experienced during childhood (Heinonen et al., 2006). Problems with mood and related coping behavior are most likely more complex, less stable and more depending on circulating steroids in interaction with the MR. Nevertheless, the results are very important for psychiatric research, knowing that depression strikes women twice as often compared to men (Bijl et al., 1998).

5' *MR* gene variants modulate psychological resilience to depression only in women, which is interesting considering the two times higher prevalence of depression among women as compared to men.

Interaction with antidepressants

Why an antidepressant drug is highly effective in one patient while it is less effective or even without efficacy in other patients is a central question in psychiatric research. Moreover, many patients experience adverse drug reactions. Regarding efficacy, one candidate gene is the multidrug resistance 1 (*MDR1*) P glycoprotein (Pgp). SNPs were identified in this transporter localized in the blood-brain barrier that affected the penetration of antidepressants into the brain (Uhr et al., 2008). Nevertheless, the molecular mechanism of antidepressant action is still poorly understood. While traditionally a link with mono-aminergic systems is suspected, other preclinical and clinical studies suggest that antidepressant drugs may induce MR and GR expression in a manner that precedes HPA normalization (Seckl and Fink, 1992; Barden et al., 1995; Bjartmar et al., 2000; Nickel et al., 2003; Zobel et al., 2004). These effects on the HPA axis may very well depend on a patient's genetic makeup, especially since we found multiple SNPs in the *MR* gene promoter region. In other studies using the NESDA cohort specifically tricyclic antidepressants (TCAs) were found to affect the CAR, while SSRIs were said to have no effect (Vreeburg et al., 2009a). However, our results show that SSRIs do seem to modulate circulating morning cortisol but depending on the patient's *MR* genotype; among the patients that were frequently using SSRIs a strong interaction effect with the *MR* -2G/C SNP on the CAR was observed. These findings point to a *MR* genotype depending effect of antidepressants.

Hence, it would be important to examine the importance of *MR* genotyping for treatment selection.

The *MR* gene might be a significant modulator of antidepressant effects.

Gene-environment interaction effect

Genetic association studies in psychiatry often give conflicting results. A clear example is the serotonin transporter gene (*5-HTT*) and its role in depression. The relatively low-expressing short (S) allele of the serotonin transporter linked polymorphic region (5-HTTLPR) was reported to associate with more symptoms of depression, while other studies found an association for the long (L) allele or no association at all (Lotrich and Pollock, 2004). These inconsistent results are in part explained by gene-environment interactions (Caspi et al., 2003), but still other factors like sex seem to moderate the relation (Risch et al., 2009). Moreover, recently it was pointed out that the serotonin transporter belongs to a class of 'plasticity genes' that also includes the monoamine oxidase-A (*MAOA*) and the dopamine D4 receptor (*DRD4*) and which render an individual more susceptible to adverse conditions, but simultaneously provide a benefit under supportive experiences (Belsky et al., 2009). The *GR* may also be considered as a prime example of a plasticity gene, mediating the effect of cortisol on susceptibility for environmental inputs. Over- and under stimulation of the GR by cortisol is since long known to enhance susceptibility to stress and stress-related disorders. These changes may be imposed by adverse early-life events causing changes in methylation of the *GR* promoter region (Weaver et al., 2004; Bet et al., 2009; McGowan et al., 2009).

The question arises whether the *MR* also functions as a plasticity gene with the effects of the *MR* haplotypes depending on interactions with environmental factors that may have induced epigenetic changes. By definition the MR modulates responses to stress as it binds cortisol and mediates its effects on the maintenance of homeostasis, with the GR acting in a complementary fashion. However, even though we did not have the opportunity to control for stressful life events in all association studies that we performed, the present data suggest that the impact of the *MR* haplotypes may not depend that much on severe stress. First of all, no data on life events were available for the Arnhem elderly cohort (**Chapter 4**) or the NTR (**Chapter 7**), but still associations were found with a specific personality trait (dispositional optimism) or disorder (MDD) that are in line; *MR* haplotype 2 confers increased levels of optimism and decreases the risk of depression in women. Still, this does not mean that an interaction between the haplotypes and stress was not there. Second, in two other studies we did control for potential gene-environment (stress) interaction but did not find proof for this. No interaction effect was found between the *MR* haplotypes and childhood trauma on cognitive reactivity (**Chapter 5**). Also no interaction effect was found

between the *MR* -2G/C SNP and childhood trauma or adverse life events on the CAR (**Chapter 6**). We did however find a non-significant trend for an interaction effect between the *MR* I180V SNP and trauma on the CAR (data not shown). Furthermore, previous studies by our group and by others did identify clear interaction effects between the *MR* genotype and stress on neuroendocrine and behavioral response to an experimental stressor (DeRijk et al., 2006; Bogdan et al., 2010; van Leeuwen et al., 2011). Possibly, the *MR* genotype results in differential physiological and psychological functioning already with less severe stress like daily hassles. Larger experimental groups and longitudinal studies are necessary for reaching a final conclusion on the potential interaction effects between the *MR* gene and stressful events on psychological health. Finally, differences in methylation patterns can still be tested in the brain tissue samples described in **Chapter 2**, although no data on trauma or life events are available and no difference in methylation was detected for the *GR* promoter region (Alt et al., 2010). Again, larger studies including data on stressful life events are warranted.

Possibly the 5' *MR* gene variants result in significant differences in human physiological and psychological wellbeing already under mild stress, like daily hassles.

Conclusion

The previous and present data indicate that the *MR* gene is an important modulator of human physiological and psychological wellbeing. Most associations that were found were gender-specific or exclusively found in women. In addition, important interactions were found with antidepressant usage and experimental stress.

8.3 Implications

Extended view on *MR* gene variability

Previous reports on *MR* gene variability almost exclusively describe the effects of the common and functional -2G/C and I180V SNPs. Looking at the potential combinations of those two SNPs, three haplotypes are commonly found. Association found with these SNPs and haplotypes are potentially modulated by additional SNPs in the *MR* gene. However, the exon 2 SNPs are not linked to SNPs further downstream in the *MR* gene because of a recombination hotspot (DeRijk et al., 2011), but they are linked to multiple SNPs in the *MR* gene promoter region (**Chapter 2**). These promoter SNPs do not result in additional haplotypes, as they are almost 100% linked to the exon 2 haplotypes (**Figure 1**).

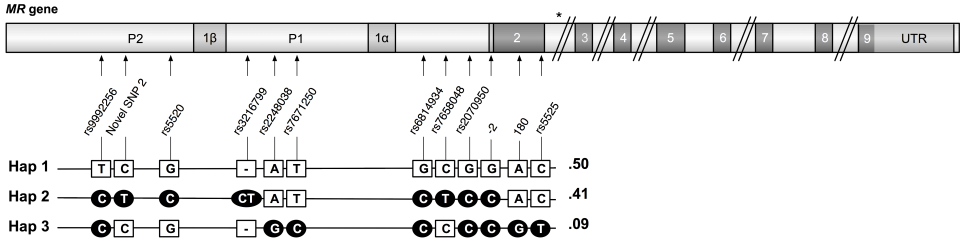

Figure 1 Schematic overview of the *MR* gene with its respective 5' SNPs and haplotypes. Nucleotide differences as compared to the most frequent haplotype 1 are indicated (black ovals). Haplotype frequencies were based on 50 anonymous blood DNA samples obtained from the general physician laboratory in Leiden. The 5' SNPs are not related to SNPs more 3' in the *MR* gene as a recombination hotspot exists in intron 2 (asterisk). Abbreviations: P1, promoter 1; P2, promoter 2; UTR, untranslated region.

This implies that effects associated with the SNPs in exon 2 are potentially at least in part due to the promoter SNPs 5' upstream. This is likely since *MR* gene expression is dependent on differential regulation and splicing of the distinct exons 1, also located in the 5' promoter region. In addition, the current results also mean that, in order to perform association studies with the 5' *MR* gene variants, genotyping of the -2G/C and I180V SNPs (or other SNPs that distinguish between *MR* haplotype 1, 2, and 3) is sufficient. Still, multiple additional SNPs exist at the 3' end of the *MR* gene (DeRijk et al., 2011), which may interact with the 5' *MR* gene variants. Moreover, in the present thesis only 4 kb of the *MR* gene promoter region was analyzed, while as yet it is not known where the *MR* promoter region actually stops. Therefore, interaction with SNPs more 5' of the gene may occur. In other words, the research described in this thesis largely extends our previous knowledge on *MR* gene variability and functioning, still the picture may not be complete.

Implications for psychiatric genetics

Multiple genes are suggested to be involved in the pathophysiology of depression, like the *5-HTT*, *BDNF* and *FKBP5* genes. Because the etiology of depression is multifactorial and therefore complicates clinical diagnosis, many association studies give conflicting results. We have been repeatedly able to show similar associations with the 5' *MR* gene variants and specific phenotypes. Collectively the present data prove that the *MR* gene can be added to this list of genes that modulate susceptibility to stress and depression. Our studies are based on extensive animal research, which resulted in valuable leads to hypothesize the MR to play a potential role in mood disorders. Together with the current results (*in vitro*, neuroendocrine, psychological) the data demonstrate that candidate gene studies are not always prone to failure. The data also disprove the notion that only genome-wide-association (GWA) studies including thousands and thousands of patients and controls have enough power to identify real genetic risk factors (Abbott, 2008). The cohorts described here range from a 150 to 3500 subjects and included individuals from the general population or real case-control study groups with varying age, but still significant and comparable associations were found. Importantly, we have shown that effects of the *MR* gene variants

can only be observed or are magnified during interaction with other factors like gender or the use of antidepressants. This may be one of the reasons why many GWA studies thus far were unsuccessful in identifying significant risk genes, including the *MR*. Future genetic association studies, including GWAs, may include more detailed interactions with demographic, health and environmental factors.

8.4 Possible mechanism for MR effects on optimism and depression

An important question is how cortisol can affect via MR the psychological risk of depression. We propose that the *MR* haplotypes modulate the cortisol effect on appraisal and behavioral flexibility and hence vulnerability for distress. Multiple studies suggest a role for the MR in appraisal and cognitive and behavioral flexibility. For example, rodents alter their escape strategy from a water maze after manipulation of MR activity (Oitzl and de Kloet, 1992). Also in humans the modulation of MR activity with antagonists results in changes in performance in cognitive tasks that are used to determine selective attention and mental flexibility (Otte et al., 2007). Here we found associations between the *MR* gene and variability in optimism, hopelessness and rumination.

The trait optimism appears to influence people's selective attention to emotional stimuli (Isaacowitz, 2005) and their coping behavior when dealing with adverse or difficult situations, for example when diagnosed with a serious disease (Carver et al., 2010b). When encountering a difficult situation, optimists show more acceptance, are more motivated to deal with the situation and use positive reframing, which seems to relate to less distress (Carver et al., 1993). Helplessness or hopelessness may be seen as less flexible, as it is related to a low motivation to take action ('giving up') when dealing with an acute stressor or even suicidal thoughts (Henkel et al., 2002; Antypa et al., 2010a). Rumination may also be seen as less flexible as people tend to think repetitively and passively about their negative emotions, potentially mediating the onset and maintenance of depression (Nolen-Hoeksema, 2000). Together the data fit with the cognitive model of depression, which would involve the precipitation of depression due to an interaction between stressful and genetic factors that results in dysfunctional attitudes, cognitive reactivity and negative cognitive bias (Beck, 2008). Indeed, improved mood by long-term antidepressant treatment seems to be preceded by an increase in positive emotional processing (Harmer et al., 2009). This may be linked to the induction of hippocampal MR expression, which was observed in rodents treated with antidepressants (Seckl and Fink, 1992; Bjartmar et al., 2000).

Possibly the *MR* gene modulates psychological risk of depression by influencing a subject's cognitive flexibility and susceptibility for distress.

Which brain circuits are underlying the influence of MR on psychological health is not completely clear. It may involve the neurobiological system important for emotional processing. Optimism was found to relate to attention bias towards positive stimuli (Isaacowitz, 2005) and enhanced activity in the amygdala regulating emotions and the rostral anterior cingulate cortex (Sharot et al., 2007). In contrast, depressed patients often show a cognitive bias towards negative emotions which would relate to reduced prefrontal function and an increase in amygdala responses to negative stimuli (Beck, 2008).

Alternatively, the influence of the MR on psychological health may involve the neurobiological system for reward and motivation, but conclusive data are lacking so far. It has been suggested that optimists have a neurobiological system for reward and motivation that is hyperactive or resistant to change (Southwick et al., 2005). In contrast, depression is often characterized by anhedonia (decreased drive and reward for pleasurable activities) and reduced motivation, which may relate to aberrant activity of the amygdala and striatum, including the nucleus accumbens (Nestler et al., 2002). Glucocorticoids are known to influence reward and motivation, although it seems to be facilitated mainly by the GR (Marinelli and Piazza, 2002; de Jong and de Kloet, 2004; Fiancette et al., 2010). It is less clear whether and how the MR plays a role, since these receptors are not highly expressed in the dopaminergic reward pathways. Yet, a first link between the *MR* gene and reward was recently presented; *MR* 180 V-allele carriers, that is haplotype 3, seem to be less able to modulate behavior as a function of reward after an acute, uncontrollable stressor (Bogdan et al., 2010). Our own data (**Chapter 2**) showed that in the depressed brain MR expression is particularly lower, compared to control brain, in the hippocampus, inferior frontal gyrus and cingulate gyrus but not in the amygdala or nucleus accumbens.

As a final question, how can the MR modulate the, sometimes, fast responses to stress (escape from water maze by rodents, human performance on cognitive tasks, appraisal processes) when the MR and GR are mainly known to act as gene transcription factors? The answer may lie in the recent discovery of a cellular membrane version of the MR. This membrane-bound MR enhances fast stress-induced glutamate release and excitability in the hippocampus and amygdala causing metaplasticity in the limbic circuitries (Karst et al., 2005, 2010). This discovery provides the mechanistic underpinning of the idea of cortisol controlling via MR the initial stress reaction important for appraisal, coping and learning processes, while the GR contributes to the control of later adaptive phases important for recovery and storage of the experience in the memory, preparing an individual for the next encounter (de Kloet et al., 1999, 2005; Joels et al., 2008; Zhou et al., 2010). In the long term the MR may, through its effects on many target genes, influence the integrity of limbic brain structures and people's personality and intrinsic behavior and eventually people's psychological wellbeing.

8.5 Future perspectives

The results presented in the current thesis are in support of the hypothesis that through differences in central MR activity disturbances in cortisol effects may underlie in part the

pathophysiology of depression. Several questions are left that need to be addressed. An initial question relates to the importance of MR expression in the brain. At the molecular level it is as yet not clear how the SNPs in the *MR* promoter region affect promoter activity and therefore transcript expression. The SNPs are predicted to affect binding of multiple transcription factors. However, it is also possible that they influence epigenetic modifications, like DNA methylation. Electrophoretic mobility shifting assays and DNA methylation analysis could clarify this. In addition, testing whether the *MR* SNPs, particularly the promoter SNPs, truly interact with sex steroids or antidepressants and hereby influence MR expression could lead to new treatment approaches. Additional *in vitro* studies could clarify this. In this respect, determining the *in vivo* influence of *MR* haplotypes in the limbic brain would further clarify the importance and dynamics of MR expression, while taking into account also the sex-specific aspects. However, this would be a challenging study, as it would demand a substantial number of *postmortem* brain samples in order to find a significant association. Finally, additional SNPs may be located more 5' of the *MR* promoter region while several SNPs have been identified at the 3' end of the *MR* gene (DeRijk et al., 2011). It is possible that these SNPs modulate the effect presented here and in previous studies. The same is true for other genetic (gene-by-gene interaction) and environmental (gene-by-environment) factors. Accordingly, the abovementioned studies will shed light on the mechanisms underlying inter-individual differences in MR expression.

Another question relates to the importance and implications of the MR in HPA regulation. We found that *MR* SNPs and haplotypes can have substantial effects on HPA regulation, particularly in a specific context. In the present thesis we have seen that the *MR* genotype interacts with commonly used antidepressants (SSRIs) in its effect on the cortisol awakening response (CAR) in depressed patients (**Chapter 6**). Some patients showed a heightened and prolonged CAR, while others showed a completely flattened CAR. One may wonder whether this is desirable, since this gene-by-SSRI interaction effect on HPA axis reactivity might influence psychological health. Additional studies are necessary to elucidate further which factors magnify the *MR* genotype effects on HPA regulation. This would demand a high-throughput and very precise sampling system, as HPA reactivity shows, because of its ultradian rhythmicity, hourly changes in responsiveness (Lightman and Conway-Campbell, 2010).

Most intriguingly are the data demonstrating that the *MR* haplotypes associate with differential psychological health, but predominantly in the women. The *MR* haplotypes do not seem to modulate the personality and risk of depression of men. Among women, *MR* haplotype 2 carriers seem to be more resilient to depression. This is interesting, as the prevalence of depression is two times higher in women compared to men (Bijl et al., 1998). A key question is through which mechanisms this gender-specific genetic vulnerability are modulated. The data described in this thesis suggest that the dynamics of MR expression in the brain is important. As a start, challenge studies with MR specific ligands combined with psychological assessments may give an initial answer towards the importance of enhanced MR activity as a protective factor. Second, we would predict that interventions to prevent psychopathology would bias women that are carriers of one or two haplotypes 1 or 3. One way would be through psychotherapy, possibly in combination with MR stimulation. As an

example, an increase in an individual's level of optimism seems achievable (Fosnaugh et al., 2009) and could potentially reduce an individual's level of distress and risk of psychopathology. Still, longitudinal studies are warranted to assess which *MR* haplotypes truly interact with (stressful) environmental factors in their effect on psychological wellbeing.

With respect to current pharmacological treatment, knowing a patient's *MR* genotype seems relevant for antidepressant selection as we found an SSRI-by-*MR* genotype interaction effect on the CAR. The use of patient-specific induced pluripotent stem cells (iPSCs) may give new leads for *MR* genotype dependent neurobiological and clinical effects of antidepressants. In addition, the use of iPSCs may help finding effective MR specific compounds. However, longitudinal cohort studies are necessary to find out what the actual clinical effects are of the different antidepressant compounds depending on the *MR* genotype. The results may facilitate personalized treatment, that is, based on the woman's genotype the clinician may select the appropriate pharmacological compound and dose.

With respect to specific modulation of MR activity it is important to keep in mind that the MR is a promiscuous receptor that is not only expressed in the brain but also in for example the kidney, heart, arteries and colon. In the kidney it specifically binds aldosterone instead of cortisol because of the cortisol-inactivating enzyme 11β-HSD2 and regulates blood pressure (Seckl, 1997; Funder, 2005). Previous research by our group and others demonstrated that the *MR* SNPs described here indeed also associate with variability in heart rate and blood pressure and with hypertension (see **Table 1**). Targeting the MR by for instance intravenous injection will potentially lead to severe side effects. Therefore a method to target the MR in specific brain regions is warranted.

Collectively the results presented in this thesis suggest that the brain MR is a susceptibility factor for the pathogenesis of stress-related mental disorders. This implies that optimal MR functioning (relative to GR) is associated with resilience and mental health, while both hypofunction and hyperfunction of the MR enhances vulnerability to stressful environmental challenges and cognitive inputs (**Figure 2**).

Depression seems to be associated with hypofunction of the MR, which is attenuated by the *MR* gene haplotype 2 variant potentially providing an enhanced MR function. Also MR hyperfunction is known to be damaging, as is particularly evident from endorgan damage (kidney, heart, brain) under conditions of hypertension and osmotic imbalance. The latter very threatening clinical condition is rescued by blocking the MR with an antagonist. In depression, the MR is thought to play a role in the response to antidepressants, which is in part based on the finding that MR expression can be induced by TCAs and SSRIs. This condition is assumed to benefit from co-treatment with MR agonists and indeed recently evidence has been obtained supporting this claim (Otte et al., 2010).

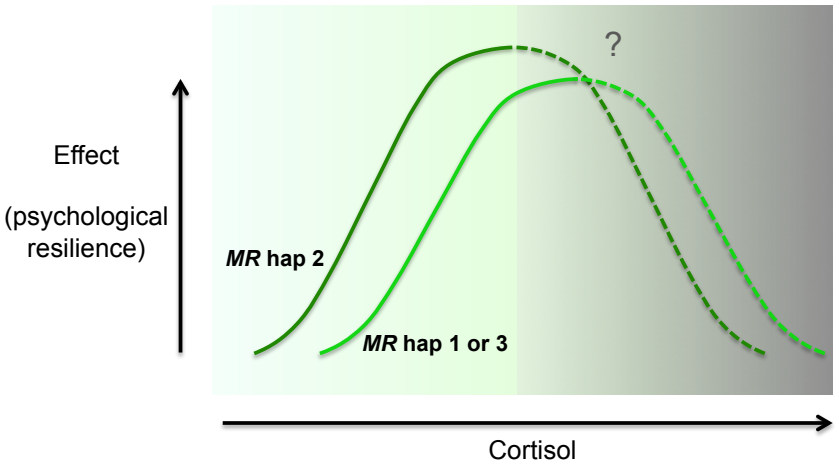

Figure 2 Proposed role of the *MR* haplotypes 1, 2 and 3 in the effect of cortisol on psychological resilience. **Solid lines** *In vitro* data indicate that *MR* haplotype 2 results in higher MR molecular activity compared to the haplotypes 1 and 3 (van Leeuwen et al., 2011; Chapter 3). Therefore, already under lower cortisol concentrations *MR* haplotype 2 will have an effect on downstream mechanisms (for example psychological resilience) compared to haplotypes 1 and 3 (curve of *MR* hap 2 shifted to the left). Moreover, the maximum effect established by haplotype 2 in comparison to haplotypes 1 and 3 may be higher (curve peak higher). **Dashed lines** The literature indicates that the dose-response relationship of cortisol and its receptors may follow an (inverted) u-shape curve (Joëls, 2006; Wardenaar et al., 2011). The dashed lines were therefore extrapolated from the solid lines. The question remains whether and at what cortisol concentration the *MR* haplotypes result in MR hyperactivity and enhanced risk of psychological disturbances. Other factors may influence the dose-response relationship of cortisol and the *MR* haplotypes, like female sex steroids.

8.6 General conclusions

Abundant evidence points to the MR as a key player in resilience and to MR activation in the limbic brain as a potential antidepressant strategy. The data presented in this thesis demonstrate that the *MR* gene is an important determinant of psychological wellbeing with the potential to modulate vulnerability for depression (see **Table 2** for an overview on the relation between the MR, resilience and depression). We have shown that MR expression is lower in several critical limbic brain regions of depressed patients. Moreover, we have repeatedly been able to show similar associations between a *MR* gene variant, haplotype 2, and a lower (psychological) risk of depression in women. The fact that the *MR* gene seems to modulate the psychological risk of depression predominantly in women is fascinating considering the two times higher female prevalence of depression. Recent findings are uncovering how MR mediated effects in the brain rapidly can modify excitatory transmission and information processing in the limbic brain (Karst et al., 2005, 2010).

Table 2 Multiple lines of evidence pointing to a relation between the MR, resilience and depression

Support for a relation between the MR, resilience and depression	Reference
MR antagonist influences appraisal, behavioral response selection in rodents	Oitzl and de Kloet, 1992
Antidepressants induce MR expression	Seckl and Fink, 1992; Bjartmar et al., 2000
MR antagonist suppresses antidepressant efficacy	Holsboer, 1999
MR agonist enhances antidepressant efficacy	Otte et al., 2010
MR antagonist influences selective attention, mental flexibility	Otte et al., 2007
MR mRNA expression is lower in the limbic brain of depressed patients	Chapter 2
MR 180 V-allele (haplotype 3) associates with feelings of depression in elderly	Kuningas et al., 2007
MR haplotype 2 is associated with heightened levels of dispositional optimism in women	Chapter 4
MR haplotype 2 is associated with fewer thoughts of hopelessness during sad mood in women	Chapter 5
MR haplotype 2 is associated with less rumination during sad mood in women	Chaper 5
MR haplotype 2 associates with a lower risk for depression in women, particularly in women not using oral contraceptives	Chaper 5, 7
The *MR* -2 C-allele (and haplotype 2) associates with a lower CAR in depressed patients using SSRIs	Chapter 6

Moreover, the data presented in this thesis imply that as a function of *MR* genotype individuals may react differently to their environment at the neuroendocrine, psychological and behavioral level, potentially resulting in differences in resilience against psychopathology. Importantly, the *MR* genotype also modulates effects of antidepressants, which potentially makes it a relevant biological marker for treatment selection.

Hence my final conclusions of this thesis research are:

- The MR is an important determinant of susceptibility to stress and stress-related disorders such as depression
- The *MR* genotype is an important biological marker and target for treatment of depression, particularly in women.