The glucocorticoid receptor: transcriptional regulation and epigenetic programming

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General Abstract

Stress represents a significant problem for Western societies inducing costs as high as 3-4 % of the European gross national products, a burden that is continually increasing (WHO Briefing, EUR/04/5047810/B6). The classical stress response system is the hypothalamic-pituitary-adrenal (HPA) axis which acts to restore homeostasis after disturbances. Two major components within the HPA axis system are the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Cortisol, released from the adrenal glands at the end of the HPA axis, binds to MRs and with a 10 fold lower affinity to GRs. Both, impairment of the HPA axis and an imbalance in the MR/GR ratio enhances the risk for infection, inflammation and stress related psychiatric disorders.

Major depressive disorder (MDD) is characterised by a variety of symptoms, however, one of the most consistent findings is the hyperactivity of the HPA axis. This may be the result of lower numbers or reduced activity of GRs and MRs. The *GR* gene consists of multiple alternative first exons resulting in different *GR* mRNA transcripts whereas for the *MR* only two first exons are known to date. Both, the human *GR* promoter 1F and the homologue rat *Gr* promoter 1_7 seem to be susceptible to methylation during stressful early life events resulting in lower 1F/1_7 transcript levels. It was proposed that this is due to methylation of a NGFI-A binding site in both, the rat promoter 1_7 and the human promoter 1F.

The research presented in this thesis was undertaken to determine the differential expression and methylation patterns of *GR* and *MR* variants in multiple areas of the limbic brain system in the healthy and depressed human brain. Furthermore, the transcriptional control of the *GR* transcript 1F was investigated as expression changes of this transcript were associated with MDD, childhood abuse and early life stress. The role of NGFI-A and several other transcription factors on 1F regulation was studied *in vitro* and the effect of *Ngfi-a* overexpression on the rat *Gr* promoter 1_7 *in vivo*. The susceptibility to epigenetic programming of several *GR* promoters was investigated in MDD. In addition, changes in methylation levels have been determined in response to a single acute stressor in rodents.

Our results showed that *GR* and *MR* first exon transcripts are differentially expressed in the human brain, but this is not due to epigenetic programming. We showed that NGFI-A has no effect on endogenous 1F/1_7 expression *in vitro* and *in vivo*. We provide evidence that the transcription factor E2F1 is a major element in the transcriptional complex necessary to drive the expression of *GR* 1F transcripts. In rats, highly individual methylation patterns in the paraventricular nucleus of the hypothalamus (PVN) suggest that this is not related to the stressor but can rather be interpreted as pre-existing differences. In contrast, the hippocampus showed a much more uniform epigenetic status, but still is susceptible to epigenetic modification even after a single acute stress suggesting a differential 'state' versus 'trait' regulation of the *GR* gene in different brain regions.

The results of this thesis have given further insight in the complex transcriptional regulation of *GR* and *MR* first exons in health and disease. Epigenetic programming of *GR* promoters seems to be involved in early life stress and acute stress in adult rats; however, the susceptibility to methylation in response to stress seems to vary between brain regions.

Zusammenfassung

Stressbedingte Krankheiten stellen ein beträchtliches Problem für westliche Industriestaaten dar. Die durch Stress verursachten Kosten belaufen sich in Europa auf etwa 3-4 % des Bruttosozialproduktes und werden vermutlich stetig ansteigen (WHO, EUR/04/5047810/B6). Die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) stellt das klassische System der Stressantwort beim Menschen dar, da sie die Homöostase verschiedener Zell-und Organfunktionen unter Stress wiederherstellt. Die beiden wichtigsten Vermittler der Stressantwort sind der Glukokortikoid-Rezeptor (GR) und der Mineralokortikoid-Rezeptor (MR). Das Stresshormon Kortisol, welches am Ende der HPA-Achse von den Nebennieren ausgeschüttet wird, bindet an den MR und mit einer 10-fach geringeren Affinität auch an den GR. Sowohl eine Beeinträchtigung der HPA-Achse als auch ein Ungleichgewicht im MR/GR Verhältnis kann das Risiko für Infektionen, Entzündungen und stressbedingte, psychiatrische Erkrankungen erhöhen.

Depressionen sind durch eine Vielzahl an Symptomen gekennzeichnet, der einheitlichste Befund ist jedoch die Hyperaktivität der HPA-Achse. Die Ursache hierfür könnte eine geringere Anzahl oder eine verminderte Funktion von GRs und MRs sein. Das *GR* Gen kodiert mehrere alternative Erstexone, was in unterschiedlichen *GR* mRNA Transkripten resultiert, während für den *MR* zur Zeit nur zwei alternative Erstexone bekannt sind. Sowohl der humane *GR* Promotor 1F als auch der homologe Promotor 1_7 der Ratte sind anfällig für Methylierung durch frühkindlichen Stress, was in geringeren 1F/1_7 Transkriptmengen resultiert. Vorangegangene Studien stellten die Hypothese auf, dass dies auf die Methylierung einer NGFI-A Bindestelle zurückzuführen ist, im Promotor 1_7 der Ratte und im humanen Promotor 1F.

Das Ziel dieser These war es, die differenziellen Expressions- und Methylierungsmuster der verschiedenen *GR* und *MR* Varianten in mehreren Hirnregionen des limbischen Systems, sowohl in gesunden Kontrollen als auch in depressiven Patienten, zu bestimmen. Des Weiteren wurde die transkriptionelle Kontrolle des *GR* Transkripts 1F untersucht, dessen Expression bei Depressionen, Kindesmissbrauch und frühkindlichem Stress verändert ist. Dabei wurde die Rolle von NGFI-A und anderen Transkriptionsfaktoren auf die Regulation der 1F Transkripte *in vitro* untersucht sowie

der Effekt einer Überexpression von *Ngfi-a* auf den Ratten-Promotor 1_7 *in vivo*. Zudem wurde die Anfälligkeit der verschiedenen *GR* Promotoren auf epigenetische Veränderungen bei Depressionen analysiert. Der Effekt von akutem Stress auf den Methylierungsgehalt im Promotor 1_7 wurde in Tierexperimenten erforscht.

Die Ergebnisse weisen darauf hin, dass die *GR* und *MR* Erstexone im humanen Gehirn unterschiedlich exprimiert werden und dass dies nicht auf epigenetische Veränderungen in den Promotoren zurückzuführen ist. Es konnte gezeigt werden, dass NGFI-A keinen Effekt auf die endogene Expression von 1F /1_7 hat, weder *in vitro* noch *in vivo*. Erstmalig konnte jedoch beobachtet werden, dass der Transkriptionsfaktor E2F1 eine wichtige Rolle bei der transkriptionellen Regulation der 1F Expression spielt. Die Tierexperimente haben gezeigt, dass der Methylierungsgrad des 1_7 Promotors im paraventrikulären Nukleus des Hypothalamus sehr variabel ist. Dies deutet darauf hin, dass diese epigenetischen Veränderungen bereits im frühkindlichen Stadium auftreten, jedoch nicht nach einem einzelnen akuten Stressfaktor. Im Gegensatz dazu scheint der Hippocampus auch auf akuten Stress mit Veränderungen in der Promotor-Methylierung zu reagieren.

Die Ergebnisse, die im Rahmen dieser These gewonnen werden konnten, gewähren einen tieferen Einblick in die komplexe transkriptionelle Regulation der *GR* und *MR* Erstexone im gesunden und depressiven Gehirn. Epigenetische Veränderungen der *GR* Promotoren scheinen eine Rolle bei frühkindlichem Stress sowie akutem Stress zu spielen; diese Anfälligkeit scheint jedoch in unterschiedlichen Hirnregionen stark zu variieren.

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Erklärung

Chapter 1

General Introduction

This has been published in part in:

Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more

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General Introduction

The Hypothalamic-Pituitary-Adrenal Axis

As a response to stress or when faced with a threat, the human body tries to restore homeostasis. The classical stress response includes activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN). CRH stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary which in turn leads to the release of glucocorticoids (GC) from the adrenal glands (Figure 1). In humans, the main glucocorticoid is cortisol whereas in rodents it is corticosterone. The HPA axis does not only control peripheral functions such as metabolism, energy storage and the immune system, it also exerts profound effects on the brain (Pariante and Lightman 2008).

One important target of glucocorticoid action is the hypothalamus, which is a major controlling centre of the HPA axis. Cortisol, produced in the adrenal cortex, will negatively feedback to inhibit both the hypothalamus and the pituitary gland to maintain both basal and stress-related homeostasis (Eskandari and Sternberg 2002; Webster, J. I. et al. 2002). In addition to stress-dependent activation, Cortisol and corticosterone are released in a pulsatile ultradian pattern which defines the normal circadian rhythm (Lightman 2008).

In the brain, cortisol acts at two types of receptor, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet 1985) which are expressed by many different types of neurons. GRs are expressed everywhere in the brain but are most abundant in hypothalamic CRH neurons and pituitary corticotropes (De Kloet et al. 1998). Aldosterone-selective MRs are expressed in the hypothalamus but their highest expression was found in the hippocampus, a brain area involved in learning and memory. It is important to note that hippocampal MRs are not aldosterone-selective and bind with high affinity to GCs, approximately 10 fold higher than GRs (De Kloet et al. 1975; Veldhuis et al. 1982). Therefore, it has been postulated that the MR/GR-balance is

critical for maintaining homeostasis (De Kloet et al. 1998). MRs are thought to control the stress system activity and to mediate effects from tonic GCs, whereas GRs are involved in restoring homeostasis after disturbances and in the feedback actions to the HPA axis system (De Kloet et al. 1998). Both receptors also play a role in cognitive performance. MR activation is essential for the acquisition of new memories whereas GRs are involved in the consolidation of learned information (de Kloet et al. 1999).

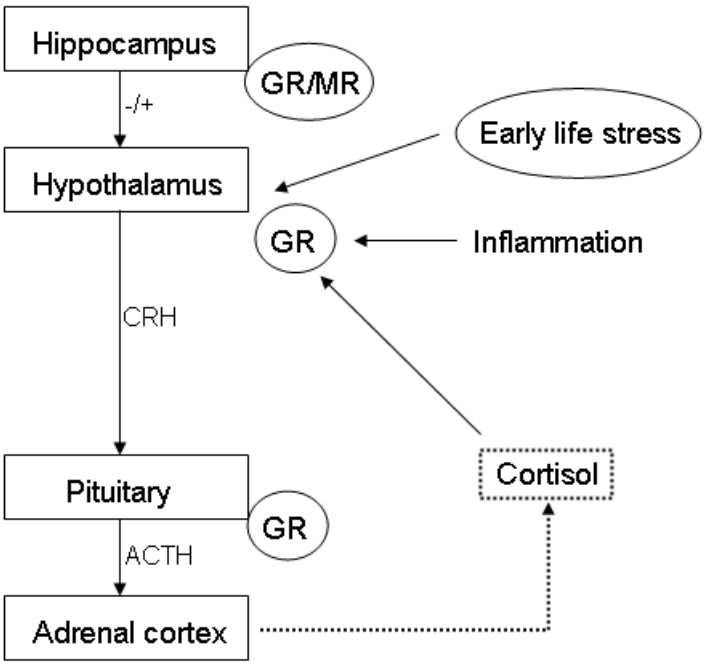

Figure 1:

Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis and biological mechanisms that lead to hyperactivity, adapted from (Pariante and Lightman 2008). Circulating cortisol (dashed line) can bind to GRs outside the brain (e.g. pituitary) or inside the brain (e.g. hippocampus and hypothalamus). Activated GRs can induce feedback inhibition to reduce the activity of the HPA axis. Environmental mechanisms such as early life stress and inflammation can affect GR function and in turn impair this feedback inhibition.

Depression: a disease with altered HPA-axis activity

Major depressive disorder (MDD) is a mental disorder characterised by low mood, decreased energy, fatigue, difficulties in thinking, concentrating, remembering or making decisions. One of the most consistent findings in major depression is that a significant number of patients have increased cortisol levels (Pariante 2003; Nemeroff et al. 2006; Pariante and Lightman 2008). This HPA axis hyperactivity is thought to be related to impaired negative feedback by endogenous glucocorticoids (Pariante and Miller 2001; Nemeroff et al. 2006). Impaired feedback regulation may be due to a lower sensitivity to GCs or a reduction in GR levels in the hypothalamus and the hippocampus. It has been shown that GR function is reduced in MDD patients, either in peripheral blood mononuclear cells or post-mortem brain tissue (Lopez et al. 1998; Webster and Carlstedt-Duke 2002). As mentioned above, the GR has a lower affinity for endogenous GCs than the MR, but a higher affinity for synthetic GCs such as dexamethasone. Therefore the GR is considered to be more important in the stress response when glucocorticoid levels are high (De Kloet et al. 1998). Reduced *GR* mRNA levels have also been found in other psychiatric disorders such as schizophrenia and bipolar disorder (Webster, M. J. et al. 2002; Perlman et al. 2004). However, MR mediated feedback inhibition seems to be intact in depressed patients (Young et al. 2003; Juruena et al. 2006). *MR* expression levels are rather unchanged or even increased in psychiatric disorders (Xing et al. 2004; Wang et al. 2008).

Hyperactivity of the HPA axis might reflect a susceptibility through early life events since these can affect GR levels. Animal studies have shown that maternal separation for longer periods resulted in an increased HPA axis activity and persisted into adulthood (Sanchez et al. 2001). Also, decreased levels of *GR* expression in the hippocampus and enhanced GC feedback sensitivity were found in the adult offspring of high caring mothers compared to those receiving low maternal care (Liu et al. 1997; Francis et al. 1999). In humans, an enhanced HPA axis activation and long-term dysregulation was found in women who suffered from sexual or physical childhood abuse (Weiss et al. 1999; Heim and Nemeroff 2002). In suicide victims with a history of childhood abuse, *GR* mRNA expression levels were reduced in the hippocampus compared to controls (McGowan et al. 2009). Thus, it has been suggested that the differences of hippocampal *GR* expression observed in adulthood may reflect the effect of early life experience on individual differences in the HPA axis response to stress (Weaver et al. 2004).

Stress-responsive neurocircuitries

The central control of glucocorticoid secretion is mediated by the paraventricular nucleus of the hypothalamus (PVN). In response to stress, CRH is released from neurons in the PVN. Lesion studies in rodents showed reduced CRH levels in the PVN and subsequent decreased corticosterone secretion (Makara 1992). Chronic exposure to stress increases *CRH* mRNA levels significantly (Sawchenko *et al.* 1993), also in humans with depression and Alzheimer's disease (Raadsheer *et al.* 1994; Raadsheer *et al.* 1995). The initial stress response is mediated by limbic brain areas, such as the amygdala, the hippocampus and the prefrontal cortex and then communicated to trans-synaptically afferents to the neurons of the PVN (de Kloet *et al.* 2009). The amygdala is known to drive behavioural and cardio-vascular responses to stress (Davis 1992). Lesion studies also showed an impact of the amygdala on the activity of the HPA axis resulting in decreased corticosterone and ACTH secretion (Allen and Allen 1974; Allen and Allen 1975). The hippocampus which displays the highest level of glucocorticoid binding and *GR* and *MR* mRNA plays an important role in HPA responsiveness. Also, an inhibitory role has been suggested as damage to the hippocampus resulted in increased glucocorticoid secretion and an increase in *CRH* mRNA in the PVN (Herman *et al.* 1989; Jacobson and Sapolsky 1991; Sapolsky *et al.* 1991). The prefrontal cortex has been shown to play a role in inhibition to the PVN and negative HPA axis feedback regulation (Herman and Cullinan 1997). Alterations in the limbic brain system have been associated with a dysregulation of the HPA axis. Changes in activity and volume of the amygdala, the hippocampus and the prefrontal cortex have been linked to major depression (Drevets *et al.* 1992; Sapolsky 1996) which is characterised by hyperactivity of the HPA axis.

Effects of acute and chronic stress on the HPA axis

The central mechanism of the neuroendocrine stress response may change when an acute stressor becomes repeated or chronic. In response to acute stress, the PVN receives input from the limbic system and the brain stem resulting in secretion of CRH activating the HPA axis. In contrast, under chronic stress, *CRH* mRNA was reduced in the PVN (Lightman 2008). Acute stress also leads to an increase in *5-HT7* receptor

mRNA in the hippocampus. This serotonin receptor subtype, that has been shown to be implicated in circadian rhythm and affective disorders, has a high affinity for antipsychotic and antidepressant drugs and was largely unaffected by chronic stress. On the other hand, *GR* and *MR* mRNA expression was only slightly affected by acute stress but significantly decreased in the rat hippocampus after chronic stress (Yau *et al.* 2001). *MR* mRNA was decreased in all hippocampal subfields whereas *GR* mRNA was selectively decreased in the dentate gyrus (DG) in response to chronic stress.

The mineralocorticoid receptor – gene and protein structure

The human mineralocorticoid receptor gene (OMIM * 600983; *NR3C2*) is located on chromosome 4q31.2 (Fan et al. 1989) and contains 8 translated exons (2-9) and 2 untranslated alternative first exons, *MRα* and *MRβ* (Zennaro et al. 1995). A third mRNA variant (*γMR*) was detected in the rat hippocampus (Kwak et al. 1993), but not in humans so far. Although both variants are expressed in aldosterone target tissue, *MRα* is the predominant form expressed in the kidney, whereas in the hippocampus *MRα* and *MRβ* are equally expressed (Zennaro et al. 1995; Zennaro et al. 1997). The ratio between *MRα* and *MRβ* varies throughout development (Vazquez et al. 1998) whilst *MRβ* is predominately expressed during the second week after birth. *MRα* seems to be the predominant transcript variant in adulthood. The differential tissue-specific expression of *MR* variants seems to be affected by changes in the sodium concentration of the cell (Zennaro et al. 1997). Both variants are under the transcriptional control of two different distinct promoters, *P1* (1 kb) and *P2* (1.7 kb) (Zennaro et al. 1996) that are both induced by glucocorticoids. However, only the distal *P2* promoter was shown to be stimulated by aldosterone in a dose- and hMR-dependent manner. The alternative mRNA variants are translated into two protein isoforms, MRA and MRB, generated by alternative initiation of translation within the common exon 2 (Pascual-Le Tallec et al. 2004). These protein variants seem to display distinct transactivation properties *in vitro* and may play a role in the tissue-specific aldosterone responsiveness. The link between the alternative first exon usage and the ATG start codons is currently unknown. Alternative splicing skipping exons 5 and 6 results in another MR protein isoform, hMRΔ5,6 (Zennaro et al. 2001). Although this 75 kDa protein lacks the ligand-binding domain (LBD), it is still able to bind to DNA and therefore acts as a ligand-independent

transactivator. In the rat, another mRNA variant has been found containing a 12 bp insertion which has also been observed in human white blood cell mRNA. This insertion leads to an altered protein isoform with 4 additional amino acids in the DNA binding domain suggested to influence MR affinity for hormone response elements (Bloem et al. 1995).

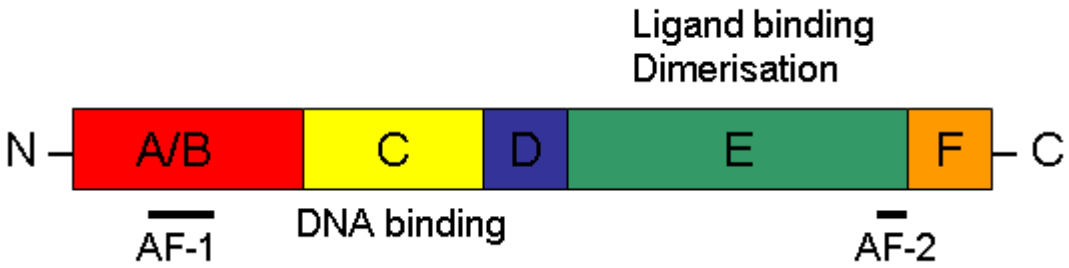

Figure 2:

Schematic representation of the mineralocorticoid receptor structure. The N-terminal domain or the A/B region contains the ligand-independant AF-1 site where coactivators can bind to the MR via a non-defined motif. The conserved DNA binding domain (DBD) or C region contains two zinc finger motifs and is responsible for recognition of specific DNA sequences. The variable D region links the DBD to the ligand binding domain (LBD) within the conserved E/F region where coactivators can interact with the MR via a ligand-dependant AF-2 domain.

The MR as well as the GR shares the typical structure of nuclear receptors (Figure 2). The N-terminal A/B region is most variable in size and sequence between different nuclear receptors. The MR is the longest member of the nuclear receptor superfamily of ligand-dependant transcription factors (Bookout *et al.* 2006). This domain shows promoter and cell-specific activity und may play a role in tissue specific expression. The DNA binding domain (DBD) is the most conserved domain and is able to recognise specific DNA sequences in target genes and required for high-affinity DNA binding (Aranda and Pascual 2001). The D region is not well conserved and serves as a link between the DBD and the LBD, allowing also rotation of the DBD. The LBD which is

required for ligand binding also mediates homo- and heterodimerisation (Aranda and Pascual 2001). Furthermore it is important for the interaction of the MR with heat-shock proteins and coregulators can interact with the MR via the LBD to transrepress target gene expression (Yang and Young 2009).

Mineralocorticoid receptor signalling

The mineralocorticoid receptor is a ligand-activated transcription factor. In absence of the ligand, the MR is localised in the cytoplasm in a complex with several chaperones such as the 90 kDa heat shock protein (HSP90) (Gomez-Sanchez et al. 2006). After ligand binding, the MR homodimerises and translocates into the nucleus where it acts on target gene expression (Figure 3). The ligand-binding domain of the MR contains an AF-2 region which includes a conserved nuclear receptor box containing at least one LXXLL (L is leucine, X is any amino acid) motif (Heery et al. 1997; Darimont et al. 1998). Several MR coactivators have been identified which can bind to this motif and either potentiate or attenuate transactivation. Two splice variants of SRC-1 (SRC-1a and SRC-1e), a member of the p160 coactivator family (Ding et al. 1998) , have been described to interact with the MR via an AF-2 site (Meijer et al. 2005). Also, other coactivators such as SRC-2 and p300/CBP interact with the MR via this site (Fuse et al. 2000; Wang et al. 2004). Coactivators can also bind the MR at the N-terminal domain via a constitutive AF-1 site that is ligand-independent (Warnmark et al. 2003).

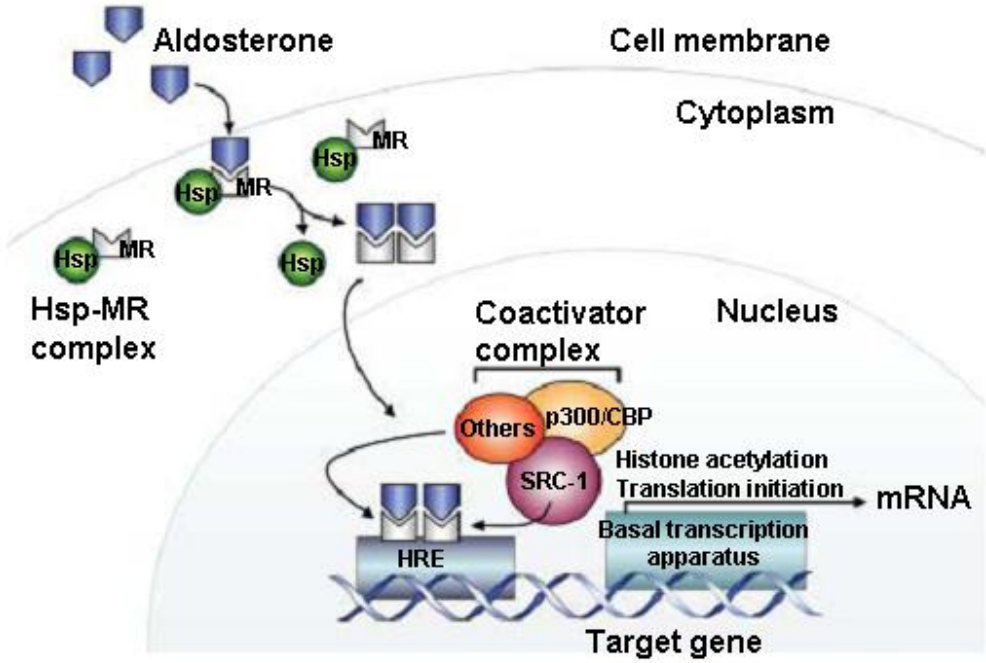

Figure 3:

Schematic representation of MR signaling upon agonist ligand binding.

The MR dissociates from Hsp, which holds it inactive in the cytoplasm, and translocates to the nucleus where it binds to a hormone response element (HRE). Coactivators, such as SRC-1 and p300/CBP, are sequentially recruited to the MR to allow histone acetylation and target gene transcription to occur. (Yang and Young 2009).

In addition to the already mentioned coactivators SRC-2 and p300/CBP, also RHA and ELL interact with the MR via this AF-1 site (Kitagawa et al. 2002; Pascual-Le Tallec et al. 2005). NCoR and SMRT have been described as the first corepressors of MR mediated transactivation via the ligand-binding domain (Wang et al. 2004). Recently, NF-YC, one of the subunits of heterotrimeric transcription factor NF-Y has also been identified to interact with the MR as a corepressor although the site of interaction with the MR is still unknown (Murai-Takeda et al. 2010). However, the number of coregulators identified for the MR is very limited compared to other nuclear receptors (Yang and Young 2009).

Structure of the *NR3C1* gene

The human *GR* gene (OMIM + 138040; *NR3C1*) is located on chromosome 5q31-q32 (Hollenberg et al. 1985) and contains 8 translated exons (2-9) and 9 untranslated alternative first exons. We and others have shown that GR levels are under the transcriptional control of a complex 5' structure of the gene, containing the untranslated first exons important for differential expression of the *GR*. All of the alternative first exons identified are located in one of the two promoter regions: the proximal or the distal promoter region**,** located approximately 5kb and 30kb upstream of the translation start site, respectively (Barrett et al. 1996; Wei and Vedeckis 1997; Breslin and Vedeckis 1998; Breslin et al. 2001; Nunez and Vedeckis 2002; Geng and Vedeckis 2004). Alternative first exons 1A, and 1I are under the control of promoters in the distal promoter region, whereas the promoters of exons 1D, 1J**,** 1E, 1B, 1F, 1C (1C1-3), 1H (Figure 4A) are located in the proximal promoter region (Turner and Muller 2005; Presul et al. 2007). Exons 1D to 1H are found in an upstream CpG island with a high sequence homology between rats and humans.

The region- or tissue-specific usage of alternative first exons leading to different *GR* mRNA transcripts (Turner and Muller 2005; Presul et al. 2007) (Figure 4B) provides a mechanism for the local fine-tuning of GR levels. Since the ATG start codon lies only in the common exon 2, this 5'mRNA heterogeneity remains untranslated, but is important for translational regulation (Pickering and Willis 2005).

Alternative mRNA transcript variants are generated by splicing of these alternative first exons to a common acceptor site in the second exon of the *GR*. Exon 2 contains an in-frame stop codon immediately upstream of the ATG start codon to ensure that this 5' heterogeneity remains untranslated, and that the sequence and structure of the GR is not affected. The *GR* also has a variable 3' region. Unlike the 5' region, the 3' variability encodes splice variants with different functions. The 3 main 3' splice variants of the *GR* are *GRα, GRβ*, and *GR-P* (Figure 4B). *GRα* and *GRβ* are generated by two alternatively spliced 3' exons, 9α and 9β. *GR-P* lacks both exons 8 and 9 and is translated into a protein with a truncated ligand binding domain (LBD) which is thought to enhance GRα activity. GRα is by far the most active form of the receptor, GRβ is thought to be a dominant negative regulator of the receptor, and little is known about the function of GR-P.

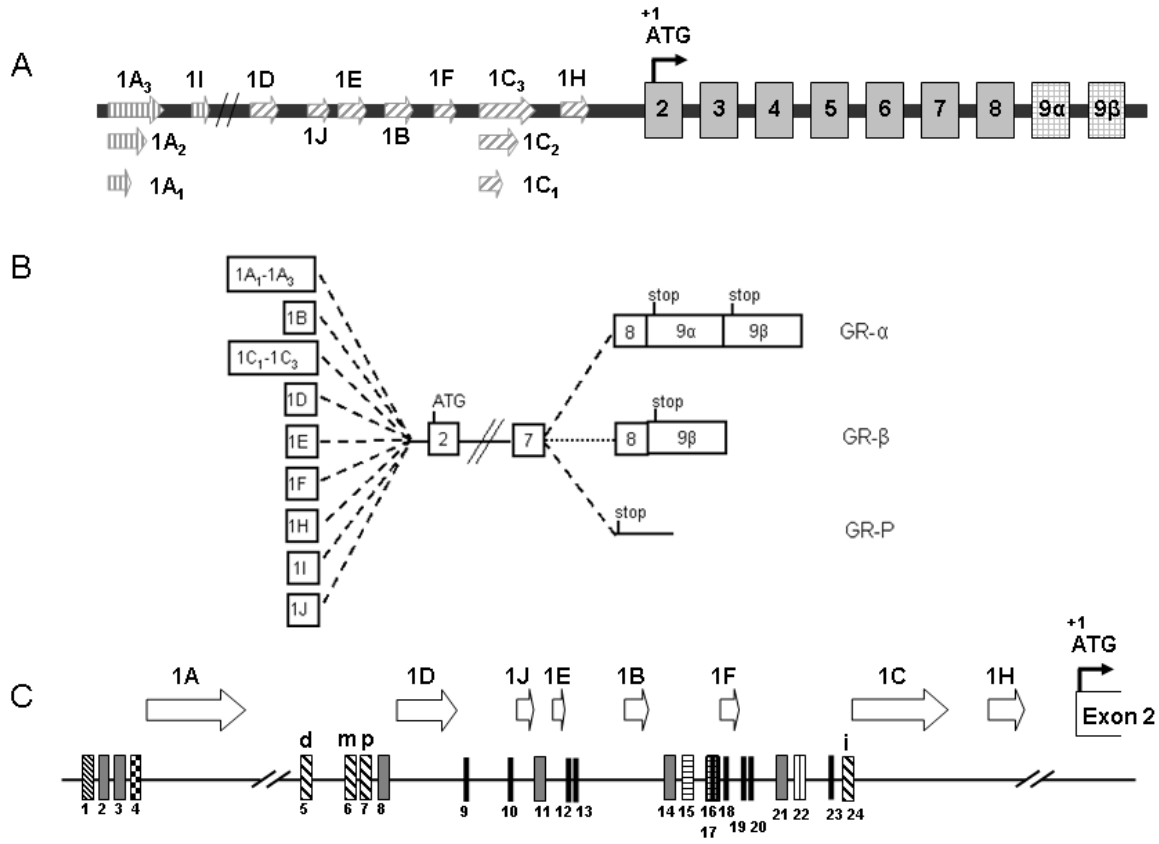

Figure 4:

Structure of the *GR* gene (*NR3C1*; OMIM + 138040; 5q31-q32), the potential mRNA transcripts and the binding sites within the CpG island.

Panel A The genomic structure of the *GR*. ▥ 5' untranslated distal exons; ▨ 5'untranslated CpG island exons; ■ Common exons; ▦ 3' alternatively spliced exons. Panel B shows the potential mRNA transcripts encoding the three GR isoforms: *GRα*, *GRβ* and *GR-P*. Panel C shows the location of the known transcription factor binding sites. ▧ IRF 1 and IRF2 (position 1); ◘ c-Myb, c-Ets ½ and PU1 (position 4); ◪ Ying Yang 1 (positions 5,6,7 and 25); ■ Glucocorticoid response elements (GRE, positions 2, 3, 8, 21, and 22); ■ Sp1 binding sites (positions 9, 10, 12, 13, 16, 19, 20, 21, and 24); □ NGFIA binding site (position 17); ▦ Glucocorticoid response factor-1 (GRF1, position 18); ▤ Ap-1 (position 15); and ▥ Ap-2 (position 23).

Alternative first exon usage and 3' splice variants

As the *GR* exhibits a large variability at the 5' end the question comes up whether this also affects splicing at the 3' end of the gene. The recent observation that transcription factors binding to pol II transcribed promoters modulate alternative splicing, supports a physical and functional link between transcription and splicing (Kornblihtt 2005). Several factors were identified that were critical for the recruitment of a specific set of co-regulators to pol II transcribed gene promoters and the production of a specific splice variants. The splice variant produced depends on the structural organisation of the gene and the nature of the co-regulators involved (Didier Auboeuf 2003). A link between transcription initiation sites and the resulting splice variant was suggested since it was shown that promoters controlled alternative splicing also via the regulation of pol II elongation rates or processivity. Slow pol II elongation paired with internal elongation pauses favoured the inclusion of alternative exons governed by an exon skipping mechanism, whereas high elongation rates of pol II, without internal pauses favoured the exclusion of such exons.

Many eukaryotic genes contain multiple promoters that are alternatively used for the production of different protein isoforms, with important physiological consequences. However, the *GR* with its variable 5' UTR, and alternative splicing in the 3' coding region is unique. Little is known about the association between the promoter usage and the resulting GR protein isoform. The 5´ UTR has tight control over local *GR* expression levels. There seems to be also a poorly understood statistical link between the 5'UTR and 3' splice variants produced. One of the first studies to address this question showed that exon 1A3, and to a lesser extend 1B and 1C contribute most to the expression of GR-α isoform (Pedersen and Vedeckis 2003). By comparing the most abundant exon 1 containing transcripts (1A, 1B 1C) with *GRα*, *GRβ*, and *GR-P* containing transcripts in different tissues and cell lines, Russcher *et al*. found a correlation between promoter usage and alternative splicing of the *GR* gene (Russcher et al. 2007). More specifically they found that the expression of *GRα* is preferentially regulated by promoter 1C, whereas 1B usage favours the expression of *GR-P* isoform. No association was found with transcripts including exon 9β or with those transcribed from 1A, suggesting that *GRβ* splicing may be associated with one of the recently identified exon 1 variants such as 1D to 1F and 1H that were not included in the above study (Russcher et al. 2007).

We also confirmed that in post-mortem brain tissues of patients with major depressive disorder (MDD) altered promoter usage influenced the resulting 3' *GR* isoform, with a negative correlation between *GR-P* expression and promoter 1B usage in all brain areas of MDD patients but not in normal control brains. A negative correlation was also found between the 1C promoter usage and *GR-P* expression in MDD brains. These results suggest that the promoters 1B or 1C do not play a significant role in *GR-P* expression in MDD, and that they were rather linked to other forms with lower expression (Alt et al. 2010). Thus, current data suggest a link between the two ends of the mRNA transcript, but there is no consensus as to the nature of this link.

Transcription factors and transcriptional control of the *GR* within the CpG island

The *hGR* was initially described as a housekeeping or constitutively expressed gene with promoters that contain multiple GC boxes and no TATA or TATA-like box (Zong et al. 1990). A wide variety of transcription factors have been identified that bind in the CpG island upstream of the gene. The description of the transcription factors active within this region is complicated by their tissue-specific usage. These transcription factors were not assigned to the different exon 1 promoters since most of this work was performed before our detailed description of the first exons in this region. The transcription factors so far identified are summarised in Table 1 and their location within the CpG island shown in Figure 4C. Initially, 11 DNAse 1 footprints representing unique transcription factor binding sites were found in the 1C to 1F region of the CpG island (-3259 to -2522 from the ATG start codon) including, one AP-2 and 5 Sp-1 binding sites were identified (Nobukuni et al. 1995).

Table 1: Transcription factor binding sites in the hGR proximal promoter region

Promoter	*TF* [a]	*N°*	*Sequences*	*Cell lines / Tissues*	*Technique* [b]	*Location* [c]		*reference*
						start	end	
Distal Promoter								
	IRF-1		GTAGAGGCGAATCACTTTCACTTCTGCTGGG	CEM-C7	FP, EMSA, RG	-34574	-34544	Breslin et al., 2001; Nunez et al., 2005
	IRF-2		GTAGAGGCGAATCACTTTCACTTCTGCTGGG	CEM-C7, Jurkat	FP, EMSA, RG	-34574	-34544	Breslin et al., 2001; Nunez et al., 2005
	GR-α		TCTGATACCAAATCACTGGACCTTA	CEM-C7	FP, EMSA	-34490	-34466	Geng et al., 2004
	GR-α		GACCGTAAAATGCGCATG	CEM-C7, IM-9	FP, EMSA, ChIP	-34436	-34419	Geng et al., 2004, 2005, 2008
	GR-β		GAGAAGGAGAAAACTTAGATCTTCTGATACCAA	CEM-C7	FP, EMSA	-34512	-34480	Breslin et al., 2001
	c-Myb		ATGTGTCCAACGGAAGCACT	CEM-C7	FP, EMSA, ChIP	-34421	-34402	Geng et al., 2004, 2005
	c-Ets 1/2		ATGTGTCCAACGGAAGCACT	CEM-C7	FP, EMSA	-34421	-34402	Geng et al., 2004
	PU.1		ATGTGTCCAACGGAAGCACT	IM-9	EMSA, ChIP	-34421	-34402	Geng et al., 2005, 2008
Proximal promoter								
1D	dYY1	1	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4807	-4799	Breslin et al., 1998
	mYY1	2	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4635	-4627	Breslin et al., 1998
	pYY1	3	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4591	-4583	Breslin et al., 1998
	GRE	4	GGCTTCCGGGACGCGCTTCCCCAATCGTCTTCAAG	Jurkat, IM-9, CEM-C7	ChIP, E	-4574	-4540	Geng et al., 2008
1J	Sp1	5	GCTGGGGCGGGGGCTT	NIH 3T3, Hela	FP, E	-4250	-4235	Breslin et al., 1998
	Sp1	6	TTCGGGGGTGGGG	Jurkat, HepG2, Hela	RG, FP, E	-4011	-3999	Nunez et al., 2002
1E	GRE	7	GTGGAAGAAGAGGTCAGGAGTTTC	Jurkat, IM-9, CEM-C7	ChIP, E	-3962	-3939	Geng et al., 2008
1B	Sp1	8	CACATTGGGCGGGAGGGG	Jurkat, HepG2, Hela	RG, FP, E	-3774	-3757	Nunez et al., 2002
	Sp1	9	TTGAACTTGGCAGGCGGCGCC	Jurkat, HepG2, Hela	RG, FP, E	-3750	-3730	Nunez et al., 2002
1F	GRE	10	GCACCGTTTCCGTGCAACCCCGTAGCCCCTTTCGAAGTGACACACT	Jurkat, IM-9, CEM-C7	ChIP, E	-3438	-3393	Geng et al., 2008
	AP-1	11	TGACACA (consensus TGAC/GTCA)	AtT-20,NIH3T3	EMSA	-3401	-3395	Breslin et al., 1996; Wei et al., 1997
	Sp1	12	TGGGCGGGGGCGGGAA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3228	-3213	Nobukuni et al., 1995
	NGFIA	13	GGGCGGGGGCGG	Rat Hippocampi / HEK293	ChIP	-3227	-3216	Weaver et al., 2004; McGowan et al., 2009
	GRF-1	14	GAAGGAGGTAGCGAGAAAAGAAACTGGAGAAACTCGGTGG	MCF7, CV-1	EMSA	-3215	-3176	LeClerc et al., 1991a, b
	Sp1	15	TCTTAACGCCGCCCCAGAGA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3172	-3153	Nobukuni et al., 1995
1C	Sp1	16	GGAGTTGGGGGCGGGGGGCG	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3107	-3088	Nobukuni et al., 1995
	Sp1	17	GCGCACCGGGCGGGGCGGCC	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3080	-3061	Nobukuni et al., 1995
	GRE	18	CTGCAGTTGCCAAGCGTCACCAACAGGTTGCATCGTTCCCC	Jurkat, IM-9, CEM-C7	ChIP, E	-2971	-2931	Geng et al., 2008
	AP-2	19	CCGCCGCGGCCCCTCGGGCGGGGA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-2923	-2901	Nobukuni et al., 1995
	Sp1	20	CGCCGTGGCGCCGCCTCCA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-2856	-2838	Nobukuni et al., 1995
	iYY1	21	CTCCTCCATTTTG	NIH 3T3, Hela	FP, D, E	-2755	-2743	Nobukuni et al., 1995

[a] TF: Transcription Factor

[b] RG: reporter gene. FP: DNAse protection / DNA footprinting. ChIP: chromation precipitation. D: deletion analysis. EMSA: electrophoretic mobility shift assay

[c] Locationswith repect to the ATG start codon in exon 2

It was initially thought that the latter transcription factors played an essential role in the basal expression of the *hGR*, although this is now less clear. Further studies identified one of the footprints in promoter 1C as a binding site for the transcription factor Yin Yang 1 (Breslin and Vedeckis 1998). YY1, expressed in a wide variety of mammalian cell types, is a zinc-finger transcription factor that can act as an activator, a repressor, or an initiator of transcription (Shrivastava and Calame 1994; Nunez and Vedeckis 2002). The same authors also revealed three other YY1 sites and another Sp1 site, initially assigned to promoter 1B. The later identification of promoter 1D suggested that these YY1 sites are probably associated with this promoter (Turner et al. 2006). Similarly, the Sp1 sites correspond to a region that was later identified as promoter 1J (Presul et al. 2007).

Similarly, several transcription factors initially assigned to promoter 1C should be reassigned to promoter 1F. AP-1, a transcription complex whose components are encoded by *c-fos* and *c-jun* proto-oncogenes binds to the AP-1 site within the *hGR* promoter 1F (Breslin and vedeckis 1996; Wei and Vedeckis 1997). This same region was also shown to bind Ku70 and Ku 80 in a tissue-specific manner (Warriar et al. 1996). Whilst most of the transcription factors identified upregulate *GR* expression, GRF-1 (glucocorticoid receptor DNA binding factor 1) has been identified as a repressor of *GR* transcription (LeClerc et al. 1991a; Leclerc et al. 1991b). At the 3' end of the rat 1_7 promoter a NGFI-A binding site was identified only 2 bp upstream of the transcription initiation site of this exon (Weaver et al. 2004). Recently, the homologous human NGFI-A binding site, together with numerous non-canonical NGFI-A sites were identified in promoter 1F of the hGR (McGowan et al. 2009).

As a transcription factor, GR also auto-regulates its own CpG island promoters. Several glucocorticoid response element (GRE) half-sites, acting in concert with c-Myb, and c-Ets protein members have been identified in promoter 1D, 1E, 1F and 1C (Geng et al. 2008).

The currently known transcription factors provide only an incomplete picture of the complex regulatory mechanisms. For instance, little is known about the proximal elements in promoters 1B and 1H. Using an *in silico* phylogenetic footprinting technique we were able to find the majority of the experimentally identified transcription factors, and predicted a wide variety of factors that are conserved between many species

(Turner et al. 2008). These are interesting candidate regulators of *GR* expression that warrant further investigations.

It has not yet been shown whether the transcription factors that bind immediately upstream of exons D, E, F, H, and I, activate the expression of these exons. Only site 13, one of 6 in the region immediately upstream of exon 1F (Figure 4C), has been shown to activate transcription of the downstream exon. Recently, it has been shown that the introns upstream of exons 1B, 1C, 1D, 1F and 1H are active promoters (Cao-Lei et al. 2011). The fact that each alternative first exon is independently controlled by its own promoter may explain the variability in tissue-specific transcriptional control of the *GR*. However, the link between the transcription factors previously identified, or predicted, and the transcription of the new CpG island first exons must be established.

Epigenetic programming of *GR* promoters

Epigenetic mechanisms such as endogenous covalent DNA modification can occur on both DNA strands at the 5' cytosine residue (Figure 5A) (Bird 2007). CpG dinucleotides are clustered in CpG island of which 29000 exist throughout the human genome (Ehrlich 2003). The amount of CpG methylation depends on the location of these CpG islands in the genome. Outside of a CpG island, CpG pairs can be methylated, however, CpG dinucleotides within seem to be protected against methylation under normal physiological conditions. Epigenetic methylation of the 5'-cytosine of a CpG dinucleotide is associated with gene silencing either by inhibition of transcription factor binding (Figure 5B) or by chromatin inactivation (Bird 2007; Meaney et al. 2007; Szyf 2009). For instance, prenatal epigenetic methylation governs genomic imprinting and inactivation of one X-chromosome (Bird 2002). The epigenetic chromatin status is sensitive to the host environment. Thus, epigenetic methylation represents a link between the environment and gene activity. In particular, early life events can have a long-lasting effect on epigenetic programming (Meaney et al. 2007; Szyf 2009). In many instances, minor changes in GR levels can have a significant impact, for example on feedback regulation of the HPA axis, where hippocampal or pituitary GR levels determine the HPA axis set-points and the response to stress.

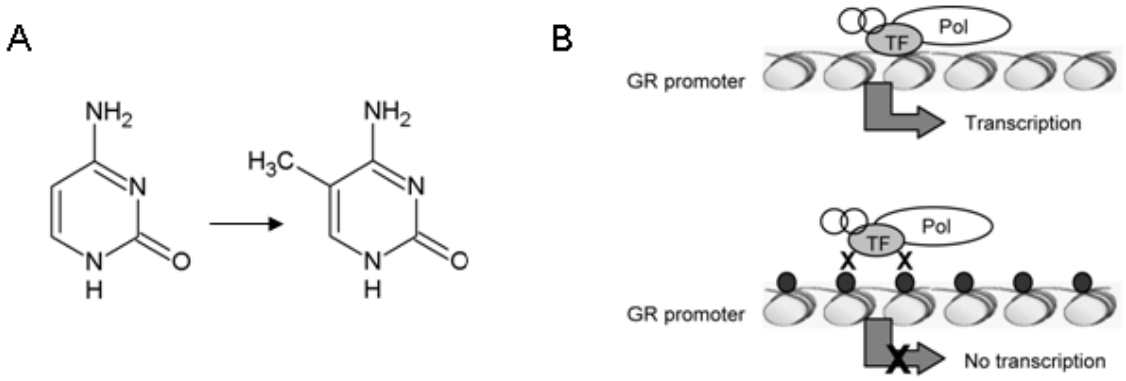

Figure 5:

DNA methylation inhibits transcriptional regulation of genes.

A: Cytosine can be methylated at carbon number 5 into 5'-Methylcytosine.

B: A complex of transcription factors (TF), the DNA polymerase (Pol) and co-activators (open circles) normally binds to the *GR* promoter and thus regulates gene expression. Methylation of the transcription factor binding site in the *GR* promoter (full ovals) can lead to inhibition of binding to the promoter resulting in reduced expression of the GR. Figure from Alt et al., 2010 with permission.

Experimentally, maternal care such as licking-grooming (LG) and arched-back nursing (ABN) has been shown to translate into epigenetic methylation of the *Gr* promoter 1_7 with profound and lasting effects on the stress response of the offspring. (Weaver 2007). The Ngfi-a binding site in the *Gr* promoter 1_7 (Figure 6), homologous to the human 1F, was highly methylated (>80%) in the offspring of low caring mothers whereas it was rarely methylated in the offspring of high caring rats (Weaver et al. 2004). As a result, binding of Ngfi-a to the *Gr* 1_7 promoter was inhibited in the hippocampus of offspring of low caring mothers and *Gr* 1_7 expression was reduced (Weaver et al. 2007). Interestingly, these effects were reversed by cross-fostering indicating a direct effect of maternal care on the epigenome of the offspring (Weaver et al. 2004). Infusion of L-methionine reversed these effects on methylation and Ngfi-a binding to the exon 1_7 promoter in the rat brain (Weaver et al. 2005).

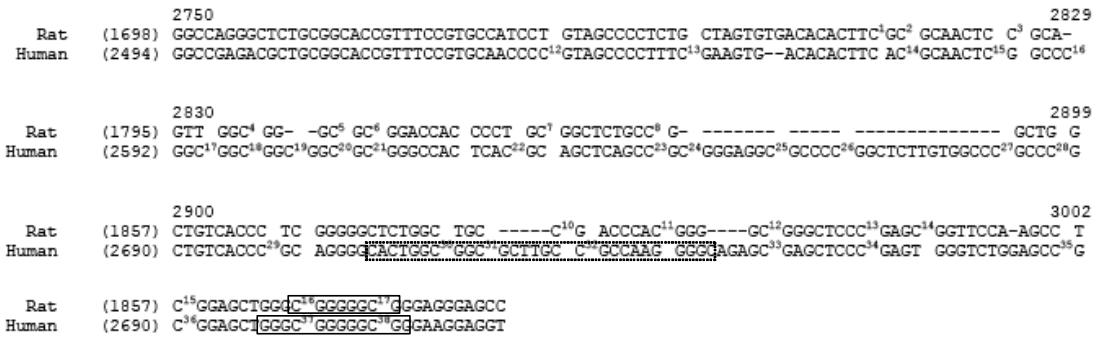

Figure 6:

Alignment of the rat *Gr* promoter 1_7 and the human *GR* promoter 1F.

Solid-lined boxes represent known canonical Ngfi-a binding sites either in the rat (Weaver et al., 2004; Daniels et al., 2009; Herbeck et al., 2010) or the human (Moser et al., 2007; Turner et al., 2008; Oberlander et al., 2008; Alt et al., 2010) promoter. The broken-lined box in the human 1F promoter represents a hypothetical non-canonical NGFI-A binding site (McGowan et al., 2009).

In Lewis and Fisher rats that naturally differ in their stress response and hippocampal Gr levels, the 1_7 promoter was shown to be un- or poorly methylated throughout (mostly below 10% and never exceeding 30%)**,** with no difference between the two strains. Feeding these rats a methyl-supplemented diet had no significant effect on the *Gr* promoter 1_7 methylation levels (Herbeck et al. 2010).

Using the maternal separation model to change the stress response in rat pups, Daniels *et al*. observed elevated *Ngfi-a* levels and significant behavioural changes. In this model, the 1_7 promoter, including both CpG sites within the Ngfi-a binding site, was uniformly un-methylated even after applying the maternal separation stressor (Daniels et al. 2009).

Epigenetic programming of the *GR* is not limited to central tissues such as the hippocampus. It has also been proposed that dietary restriction could lead to changes in DNA methyl content, affecting epigenetic programming of the *GR* promoter both centrally and peripherally (McGowan et al. 2008). Feeding a protein-restricted diet to pregnant dams lead to a hypomethylation of the major *Gr* promoter 1_{10} and to an increased expression of *Gr* in the liver of these rat pups (Lillycrop et al. 2007).

These animal models showing changes in *Gr* promoter methylation are not easily transferred to humans. Nevertheless maternal adversities like depression or protein restriction and their effects on the epigenome of offspring have been investigated (Table 2). Oberlander *et al*. showed that prenatal exposure to maternal depression leads to increased methylation levels of the *GR* promoter 1F at the NGFI-A binding site in cord blood of newborns (Oberlander et al. 2008). Like in most rat experiments, methylation levels were uniformly low (5-10 %) with small but significant differences between children of depressed and healthy mothers.

Several studies also investigated alterations in the human *GR* 1F promoter in specific disease populations based on the rat 1_7 data of Weaver *et al*. (Weaver et al. 2004). In neurological disorders such as Parkinson's disease, Alzheimer's or dementia no hypermethylation of the 1F promoter and the NGFI-A binding site could be found (Moser et al. 2007). We showed that in major depressive disorder there was no methylation of the NGFI-A binding site of the 1F promoter in several regions of human post-mortem brains (Alt et al. 2010). However, in suicide victims with a history of child abuse, McGowan *et al*. found increased methylation patterns compared to suicide victims without abuse (McGowan et al. 2009). In this study, methylation of another putative NGFI-A binding site within promoter 1F resulted in decreased expression levels of 1F transcripts and overall *GR* levels. The known NGFI-A binding site was completely unmethylated in all of the suicide victims. Thus, it is possible that other transcription factor binding sites are important for the transcriptional regulation of the *GR* 1F promoter and that these are more sensitive to epigenetic modifications. Interestingly, in all four of the above studies (Moser et al. 2007; Oberlander et al. 2008; McGowan et al. 2009; Alt et al. 2010), levels of methylation were always very low in comparison to those in the LG-ABN rats (Weaver et al. 2004). Investigating the complete *GR* CpG island, we were able to show highly variable methylation patterns among different *GR* promoters in PBMC's of healthy donors, suggesting that epigenetic programming may not be restricted to the 1F promoter, but operates throughout the CpG island (Turner et al. 2008). It remains unclear, however, what triggers changes in methylation, and when are the different tissues most susceptible to epigenetic programming. Despite some contradictions it seems that levels of methylation are consistently low in the brain, and somewhat higher and more variable at least in the blood mononuclear cells and the liver.

Table 2: Methylation analyses of GR promoter regions

GR promoter	*Species*	*Model*	*Method*	*Tissue*	*Overall methylation levels*	*CpG specific comments* †	*Reference*
1.7	r. nor.	LG-ABN	colony sequencing	hippocampus	<100%	80-100% at CpG 16-17 in Low LG-ABN 0-10% at CpG 16-17 in High LG-ABN	Weaver et al., 2004
1.7	r. nor.	met sup	colony sequencing	hippocampus	<15%	at CpG 16-17	Herbeck et al., 2010
1.7	r. nor.	maternal sep	direct sequencing	hippocampus	not detected	at CpG 16-17	Daniels et al., 2009
1.10	r. nor.	PR diet	MS-PCR	liver	unknown	relative to control	Lillycrop et al., 2007
1C	r. nor.	HV	methylation sensitive restriction enzyme PCR assay	umbilical blood	unknown	PR rats had 30% lower methylation correlation between relative methylation and DNMT1 expression	Lillycrop et al., 2007
1D	h. sap.	HV	colony sequencing	PBMCs	<50%	stochastic and unique	Turner et al., 2008
1E	h. sap.	HV	colony sequencing	PBMCs	>25%	stochastic and unique	Turner et al., 2008
1F	h. sap.	HV	colony sequencing	PBMCs	<10%	at CpG 37-38	Turner et al., 2008
1H	h. sap.	HV	colony sequencing	PBMCs	<75%	stochastic and unique	Turner et al., 2008
1F	h. sap.	Dep	pyrosequencing	cord blood	<7%	<5% at CpG 37-38	Oberlander et al., 2008
1F	h. sap.	Dep, Al, Park, Dem	MS-PCR	hippocampus	<10%	only 1 subject had visible methylation all other donors unmethylated	Moser et al., 2007
1F	h. sap.	Suicide / abuse	colony sequencing	hippocampus	<40%	0% at CpG 37-38	McGowan et al., 2009
1B	h. sap.	MDD	pyrosequencing	hippocampus	0-12%		Alt et al., 2010
1E	h. sap.	MDD	pyrosequencing	hippocampus	4-9%		Alt et al., 2010
1F	h. sap.	MDD	pyrosequencing	hippocampus	0-4%	0-2% at CpG 37-38	Alt et al., 2010
1J	h. sap.	MDD	pyrosequencing	hippocampus	0-7%		Alt et al., 2010

† all CpG numbers as in Figure 6

Abbreviations: r. nor, rattus norwegicus; h. sap, homo sapiens; LG-ABN, licking-grooming and arched-back nursing; met sup, methy-supplemented diet; maternal sep, maternal seperation; PR, protein-restricted; HV, healthy volunteer; Dep, depression; Al, Alzheimer's disease; Park, Parkinson's disease; Dem, dementia; MDD, major depressive disorder; MS-PCR, methylation-specific PCR

Research Objectives

As outlined in the previous paragraphs, the transcriptional regulation of the alternative glucocorticoid receptor first exons is highly complex and not yet fully understood. As the GR plays an important role in HPA axis regulation in response to stress, the first aim of this thesis was to determine the distribution patterns of *GR* mRNA and protein isoforms as well as *MR* variants in several regions of the limbic system in the healthy brain as well as in patients with major depressive disorder. The second objective was to investigate the transcriptional regulation of the *GR* transcript 1F as this transcript was particularly decreased in the hippocampus of MDD patients and concomitantly the expression of the transcription factor *NGFI-A* was reduced. The third objective of this thesis was to determine the susceptibility of the *GR* promoters to epigenetic programming. After investigation of methylation levels in the healthy as well as in the depressed brain, restraint stress experiments were performed in rodents to determine *in vivo* if a single acute stressor leads to changes in *Gr* promoter 1_7 methylation levels especially at the Ngfi-a binding site.

Outline

In **Chapter 2**, the differential distribution patterns of *GR* first exons and protein variants were investigated in post-mortem brain tissue of healthy subject and patients with MDD using quantitative PCR. Methylation levels of several *GR* promoters were determined by pyrosequencing as well as expression levels of transcription factors potentially binding to the *GR*. In **Chapter 3**, the expression of *MR* transcript variants has been investigated by quantitative PCR in the same samples used in Chapter 2. The ratio between *MR* and *GR* expression has been determined because an imbalance in this ratio may be involved in impaired HPA axis function in MDD. **Chapter 4** presents *in vitro* analyses on the transcriptional regulation of the *GR* transcript 1F. The role of NGFI-A and several other transcription factors predicted to bind the 1F promoter in *GR* regulation was analysed using transient transfections, reporter gene assays and chromatin immunoprecipitation in cells representing peripheral (293FT) and central (U373MG) tissue. **Chapter 5** provides results from animal studies where a restraint stress paradigm was applied to increase *Ngfi-a* expression levels and determine the effect on *Gr* 1_7 transcript levels by *in situ*

hybridisation in different brain areas. Also, the susceptibility of the 1_7 promoter to increased methylation levels after acute stress was investigated in different brain regions by pyrosequencing.

In **Chapter 6** the major findings of this thesis are discussed in a broader context and summarised. Furthermore, perspectives for future work related to the findings in this thesis are given.

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Chapter 2

Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed

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Summary

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is one of the most consistent findings in major depressive disorder (MDD). Impaired HPA feedback may be due to the lower glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) levels in the forebrain. GR levels are transcriptionally controlled by multiple untranslated alternative first exons, each with its own promoter providing a mechanism for tissue-specific fine-tuning of GR levels. Recently epigenetic methylation of these *GR* promoters was shown to modulate hippocampal *GR* levels. Here we investigate in post-mortem brain tissues whether in MDD HPA axis hyperactivity may be due to epigenetic modulation of GR transcript variants.

Levels of *GRα*, *GRβ* and *GR-P* transcripts were homogeneous throughout the limbic system, with *GRα* being the most abundant (83%), followed by *GR-P* (5-6%) while *GRβ* was barely detectable (0.02%). Among the alternative first exons, 1B and 1C were the most active, while 1E and 1J showed the lowest expression and transcript 1F expressed intermediate levels of about 1%.

In MDD, total *GR* levels were unaltered, although *GRα* was decreased in the amygdala and cingulate gyrus ($p<0.05$); transcripts containing exons 1B, 1C and 1F were lower, and 1D and1J were increased in some regions. *NGFI-A*, a transcription factor of exon 1F was down-regulated in the hippocampus of MDD patients; concomitantly exon 1F expression was reduced. Bisulphite sequencing of the alternative promoters showed low methylation levels in both MDD and control brains. Promoter 1F was uniformly unmethylated, suggesting that reduced 1F transcript levels are not linked to promoter methylation but to the observed dearth of *NGFI-A*.

Previous studies showed high methylation levels in the 1F promoter, associated with childhood abuse. Provided our donors were not abused, our results suggest that the pathomechanism of MDD is similar but nevertheless distinct from that of abuse victims, explaining the clinical similarity of both conditions and that susceptibility to depression may be either predisposed by early trauma or developed independent of such a condition. However, this should be further confirmed in dedicated studies in larger cohorts.

Keywords

glucocorticoid receptor; alternative transcripts; mRNA expression; major depressive disorder; epigenetics; pyrosequencing

Introduction

Over the last 40 years one of the most consistent biological findings in major depression psychiatry is the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Pariante and Lightman 2008). Activity of the HPA axis is normally controlled through negative feedback regulation by glucocorticoid (GR) and mineralocorticoid (MR) receptors in the forebrain (de Kloet et al. 2005). Pharmacological studies in major depressive disorder (MDD) have implicated the GR in impaired HPA axis feedback regulation (Juruena et al. 2006). Indeed, studies from the Stanley Neuropathology Consortium showed a reduced *GR* mRNA expression in the frontal cortex and the hippocampus in depression (Webster et al. 2002; Knable et al. 2004). In rodents with depression-like behaviour, the activity of forebrain GR was reduced (Boyle et al. 2005; Chourbaji et al. 2008). In some cases this has been related to early life events. For instance, in rodents, neonatal handling has been shown to modulate *GR* levels in the forebrain with effects on the HPA axis that persist into adulthood (Liu et al. 1997; Weaver et al. 2004). Similar changes were found in adults with a history of childhood abuse that committed suicide (McGowan et al. 2009). Long term disturbances of the HPA axis were also found in women that were physically or sexually abused during childhood, or men with early life trauma (Heim and Nemeroff 2002; Rinne et al. 2002; Heim et al. 2008). These early life adverse events are known to be major risk factors for developing major depressive disorder (MDD) (Bifulco et al. 1991; Brown and Anderson 1991; Jumper 1995; Kessler et al. 1997; McCauley et al. 1997; Weiss et al. 1999; Kendler et al. 2000; Widom et al. 2007). It has therefore been suggested that HPA axis hyperactivity in MDD may not be the consequence of depression, but rather is part of the pathophysiological mechanism leading to depression (Holsboer 1988; Tichomirowa et al. 2005; Pariante and Lightman 2008).

In order to understand GR expression it is important to understand its gene structure. The *GR* gene (OMIM + 138040; *NR3C1*) contains 8 translated exons (2-9) and 9 untranslated alternative first exons. Each one of these first exons has its own upstream promoter. Thus, this promoter region contains numerous transcription start sites each associated with one of several alternative first exons (Encio and Detera-Wadleigh 1991; Breslin et al. 2001). This complex promoter region, with a similar structure both in humans and rats lies in the CpG Island upstream of the *GR*. The region- or tissue-specific usage of alternative first exons leading to different *GR* mRNA transcripts (Turner and Muller 2005; Presul et al. 2007) provides a mechanism

for the local fine-tuning of *GR* levels. The lower levels of *GR* previously seen in MDD (Webster et al. 2002; Knable et al. 2004) may also be due to altered promoter usage. Since the ATG start codon lies only in the common exon 2, this 5`-mRNA heterogeneity remains untranslated, but has been implicated in translational regulation (Pickering and Willis 2005). Recent studies have identified multiple transcription factors binding to the various *GR* promoters, including YY1 (promoter 1D), Sp1 (promoter 1B), NGFI-A (promoter 1F) and the GR itself (promoter 1A, D, E) (Nunez and Vedeckis 2002; Geng and Vedeckis 2005; Geng et al. 2008; McGowan et al. 2009). The activity of some of these transcription factors is regulated by epigenetic DNA methylation of their promoter binding site (Bird 2002). DNA methylation can occur on both DNA strands at the 5'-cytosine of a CpG dinucleotide and is associated with stable gene silencing by preventing the binding of transcription factors (Bird 2007; Turner et al. 2008). Recently, we showed in peripheral blood lymphocytes that methylation patterns throughout the alternative promoters of the *GR* are highly variable in humans (Turner et al. 2008).

Previous studies of the binding site of the transcription factor NGFI-A already showed that methylation influences alternative *GR* first exon usage and long term GR activity. Binding of Ngfi-a to the rat *Gr* promoter 1_7 (the homologue of the human 1F) is sensitive to methylation. The effect of maternal care on hippocampal *Gr* expression in rodents has been associated with a specific epigenetic modification within the exon 1_7 promoter. Intensive maternal care (licking/grooming-arched back nursing) reduced methylation levels at this Ngfi-a binding site and increased 1_7 transcript levels. In contrast, pups receiving low intensity maternal care showed high levels of methylation in the 1_7 promoter and a reduced promoter activity (Weaver et al. 2004). In humans, specific methylation of the corresponding CpG dinucleotide was observed in post-mortem hippocampi of suicide victims and correlated with childhood abuse (McGowan et al. 2009). Moser *et al*. (Moser et al. 2007) investigated the NGFI-A binding site in the human exon 1F in post mortem brain tissue from elderly subjects with a variety of psychiatric disorders but found no methylation within this region. Oberlander *et al.* investigated the family inheritance of MDD in cord blood of newborns of depressed mothers and found an association between prenatal exposure to maternal depressed mood and the methylation status of the promoter of exon 1F (Oberlander et al. 2008). Thus, 1F methylation patterns would appear to be tissue, disease and species dependent.

The differential expression of any of the untranslated alternative first exons does not directly translate into a different GR protein, but there is evidence that the first exon usage influences splicing at the 3'end of the mRNA. Thus, changes in alternative first exon expression affect both the total GR level and the balance between alternative 3' splice variants. The predominant 3' splice variant is the classical glucocorticoid mediated transcription factor GRα (DeRijk et al. 2003; Hagendorf et al. 2005; Russcher et al. 2007). GRβ, another 3'-splice variant, differs from the main GRα isoform by having 50 C-terminal amino acids replaced by a unique 15 amino acid sequence affecting ligand binding and transcriptional activity (Bamberger et al. 1995; Oakley et al. 1996). A third variant, GR-P lacks exon 8 and 9 and is translated into a protein with a truncated ligand binding domain (LBD) which is thought to enhance GRα activity. The ratio of GRα/GRβ expression is a critical determinant of GC responsiveness of various tissues (Shahidi et al. 1999; Longui et al. 2000). Higher ratios correlate with glucocorticoid sensitivity, while lower ratios correlate with glucocorticoid resistance (Koga et al. 2005; Lewis-Tuffin and Cidlowski 2006). However, in the human brain the low abundance of *GRβ* suggests that this may not play an important functional role if not associated with specific conditions such as prolonged inflammation (Webster et al. 2001; DeRijk et al. 2003). Moreover, changes in GR species and expression levels will modulate the balance between the glucocorticoid and mineralocorticoid (MR) receptors (GR/MR balance). Thus, epigenetic methylation of alternative first exon promoters represents a highly individualised mechanism to fine-tune GR activity in a tissue-dependent manner influencing also the balance between GR and MR.

We hypothesised that in MDD, changes will be observed in alternative *GR* first exon usage and total *GR* expression and that these changes correlate with epigenetic modifications. Since childhood abuse is well known to increase susceptibility to depression and in suicide victims was associated with a reduced activity of the *GR* promoter 1F in the hippocampus, we investigated whether a similar pathomechanism may play a role in MDD. To exclude that other alternative exon 1 promoters may undergo epigenetic programming we compared most of the upstream promoters and multiple brain regions of the limbic system of post-mortem brains from six MDD patients and six matched controls. We find that similar to the childhood abuse study (McGowan et al. 2009) both *NGFI-A* and promoter 1F activity are reduced in the hippocampus of MDD patients; but, the underlying mechanism is different in both conditions.

Table 3: Clinicopathological information of patients with depression and control subjects

	NBB number	*Sex*	*Age (years)*	*Braak Stage*	*post-mortem delay (h:min)*	*pH CSF*	*Brain Weight (g)*	*clock time at death*	*medication taken in the past*	*medication taken in the last 3 month*	*co-morbid conditions*	*cause of death*
MDD1	07-033	M	88	2	6:37	6.26	1225	21:15	TCA	TCA	COPD	Multiple epileptic seizures
MDD2	06-011	F	60	1	4:20	ND	1080	16:10	SSRI, BZD Hal, Mo Tamoxifen	Hal	None	Legal euthanasia
MDD3	02-051	M	81	3	6:00	6.5	1345	15:30	TCA, Hal	Hal	None	Renal insufficiency
MDD4	01-074	M	45	0	7:00	6.55	1427	2:30	SSRI, BZD	SSRI	None	Brain haemorrhage
MDD5	06-026	M	70	1	7:15	6.5	1415	8:00	TCA, Hal	TCA	None	Respiratory insufficiency
MDD6	97-057	F	81	3	5:25	6.74	1313	9:05	Hal	Hal	None	Pneumonia/Dehydration
Median	**----**	**----**	**75.5**	**1.5**	**6:18**	**6.5**	**1329**	**----**	**----**	**----**		**----**
Mean SD	**----**	**----**	**70.83 ±16.04**	**1.67 ±1.21**	**6:06 ±0.05**	**6.51 ±0.17**	**1300.83 ±130.82**	**----**	**----**	**----**		**----**
HC1	01-005	F	77	1	19:45	6.21	1149	0:15	None	Mo	None	Malignant lymphoma
HC2	01-045	M	83	1	4:35	6.49	1422	19:05	None	Mo, BZD	None	Heart attack
HC3	01-069	F	68	1	5:45	6.97	1153	12:15	Tamoxifen,BZD	None	None	Legal euthanasia
HC4	01-079	F	90	3	4:45	6.5	1120	18:45	Digoxin	Digoxin	None	Heart failure after CVA
HC5	02-008	M	62	0	9:35	6.58	1175	10:00	None	None	None	Metastasized adenocarcinoma
HC6	05-034	M	56	0	14:00	7.03	1323	0:01	None	None	None	ND
Median	**----**	**----**	**72.5**	**1**	**7:40**	**6.54**	**1164**	**----**	**----**	**----**		**----**
Mean SD	**----**	**----**	**72.67 ±12.96**	**1.00 ±1.10**	**9:44 ±0.25**	**6.63 ±0.31**	**1223.67 ±120.74**	**----**	**----**	**----**		**----**

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.

Methods

Subjects

Human post-mortem brain tissues (amygdala, Amg; hippocampus, Hi; inferior prefrontal gyrus, IFG; cingulate gyrus, CgG; nucleus accumbens, Acb) from 12 donors were obtained from the Dutch Brain Bank (Netherlands Institute of Neuroscience, Amsterdam, The Netherlands) following permission from the patient or the next of kin for a brain autopsy and for the use of the brain material and clinical information for research purposes. Based on their clinical history donors were separated in two groups. Six brain samples from donors without diagnosed CNS disease or long-term psychotropic medication were used as controls. Another six samples were obtained from patients with clinically diagnosed MDD and without documented history of childhood abuse. The patients were diagnosed during their life. The diagnosis was confirmed post-mortem by a certified psychiatrist (Dr. G. Meynen) on the basis of the medical records according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition criteria (DSM-IV, American Psychiatric Association). The depression and control groups were matched for sex, age, brain weight, post-mortem delay and for pH of the cerebrospinal fluid (CSF) that is a measure of agonal state (for clinicopathological information see Table 3). The medical record did not reveal any alcohol or other drug abuse among all subjects. Detailed information on the relevant medication in the past and in the last few months before death is given in Table 3.

Preparation of cDNA from human tissue samples

Brains were dissected, snap-frozen in liquid nitrogen and stored at -80°C. Cryostat sections of 20 µm were cut (Leica Microsystems, Rijswijk, The Netherlands) and stored in trizol. Total RNA was purified from 30 sections using the RNeasy Lipid tissue kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. RNA quantity was measured on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and the quality was determined by a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity number (RIN)

was used to assess the RNA quality (scale 1–10, with 1 being the lowest and 10 being the highest RNA quality). For each sample, an equal quantity of RNA (1000 ng) was used for the synthesis of cDNA in a 50µl reaction. First-strand synthesis of total cDNA was carried out at 42°C for 30min using the iScript cDNA synthesis kit (BioRad, Richmond, CA). Several slices from each studied brain section were stained with 0.1% cresyl violet and used for histological confirmation (Figure 7B).

Oligonucleotides and RT-PCR

Specific primer sets for all genes were identified from the corresponding mRNA sequences using VectorNTi (Invitrogen, Paisley, UK). Primers and PCR conditions are shown in Supplement 1. All primers were synthesised by Eurogentec (Seraing, Belgium). Amplification of cDNA by PCR was performed in duplicates in 25µl reactions containing 20mM Tris–HCl (pH 8.4), 50mM KCl, 200mM deoxynucleoside triphosphates (dNTP), 2.5U Platinum *Taq* DNA polymerase (Invitrogen) and either 1× concentrated SYBR Green (Cambrex, Verviers, Belgium) or 100-150nM of a GR-specific Taqman probe. Thermal cycling was performed in an ABI 7500 Sequence Detector System (Applied Biosystems, Nieuwerkerk, The Netherlands). ABI conditions for SYBR Green were: one cycle 95°C, 2 min; 45 cycles each at 95°C, 20 s; annealing, 20 s; 72°C, 30 s. Amplification and detection with a Taqman probe was performed on a ABI 7500 Sequence Detector System using the following profile: 1 cycle 95°C, 20 sec and 45 cycles each at 95°C, 3 sec; annealing, 31 sec and 72°C, 30 sec. PCR products of all samples were separated on a 2% agarose gel and visualised with SYBR Safe (Invitrogen) under UV light.

Analysis of RT-PCR data

The gene specific threshold cycle (Ct) was used for semi-quantitative assessment using a stable internal reference gene. For this normalisation three reference genes,*18S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *β-actin* were included following the procedure by Schote *et al.* (Schote et al. 2007). The comparison of the three genes identified *β-actin* as the most stable housekeeping gene. As a result

β-actin was used to normalise mRNA expression in the five different brain regions, but also normalisation with *GAPDH* gave similar results. The amount of *GR* first exon mRNA normalised to *β-actin* and relative to a specific reference sample (total *GR*) was expressed as $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen 2001). PCR efficiency after 7-log dilution series reached 97-104%. Differences between groups were evaluated using the non-parametric Mann–Whitney U-test since the data showed no normal distribution, as proposed in previous studies (Wang et al. 2008). Associations were calculated using Pearson's correlation coefficient. The tests of association and methylation analyses were corrected according to Bonferroni. Statistical analysis and graphs were performed with SPSS version 16.0 (SPSS Inc., Chicago IL, USA) and SigmaPlot 9.0 (Systat Software GmbH, Erkrath, Germany), respectively. Statistical significance was considered for $p<0.05$.

Isolation of genomic DNA from human brain samples and bisulphite modification

Cryostat sections (60µm) were cut and stored at -80°C. Isolation of genomic DNA was performed on cryostat sections using the QIAamp® DNA Mini kit (Qiagen) and stored at -20°C. The EpiTect Bisulphite kit (Qiagen) converts unmethylated cytosine residues to uracil, whereas methylated cytosines remain unmodified. According to the manufacturers' protocol, the first two steps of bisulphite treatment were performed on 400 ng of genomic DNA. The thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) program comprised several incubation steps for DNA denaturation, sulfonation of unmethylated cytosine, and deamination of cytosine sulfonate to uracil sulfonate. Cycling conditions were as follows: 1st denaturation for 5 min at 99°C, 1st incubation for 25 min at 60°C, 2nd denaturation for 5 min at 99°C, 2nd incubation for 85 min at 60°C, 3rd denaturation for 5 min at 99°C, 3rd incubation for 175 min at 60°C and hold at 20°C. Alkaline uracil desulfonation and sample purification were performed using a spin column. Modified DNA was eluted and stored at -20°C until analysis.

PCR and quantification of methylation

The bisulphite-modified DNA was used to amplify promoters 1-B, 1-E, 1F and 1-J with the primers and under the conditions shown in Supplement 1. PCR was performed using 20 mM Tris HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates, 1 x SyBR Green (Cambrex, Verviers, Belgium) and 2.5 U Platinum Taq DNA polymerase (Invitrogen) on an Opticon 2 thermal cycler (MJ Research, Landgraf, The Netherlands). Cycling conditions were as follows: 95°C for 2 minutes, 40 cycles, at 95°C for 20 sec, annealing temperature for 20 sec and 72°C for 25 sec. Products of first round PCRs were used as a template for nested amplifications using the primers and conditions in Supplement 1. PCR products were commercially pyrosequenced on a Pyromark ID (Varionostic, Ulm, Germany) to allow quantitative analysis of DNA methylation patterns.

Results

Expression of *GR* isoforms

Expression levels of total *GR* showed a similar pattern throughout all analysed brain areas (Figure 7C) and there was no difference between MDD and control brains irrespective of whether exon 2 or a region spanning exons 3 and 4 (data not shown) common to all *GR* isoforms were amplified. As expected, the classical *GRα* dominated over all other isoforms with a 1500-fold higher expression than *GRβ*. In MDD a significant decrease of *GRα* (Figure 7D) was detected in the amygdala (p=0.031) and the cingulate gyrus (p=0.032). *GRβ* showed a significant decrease in the inferior prefrontal cortex (p=0.014) in patients with MDD (Figure 7E). *GR-P* showed no significant changes in expression levels in all brain regions but was slightly higher in the inferior prefrontal gyrus and the cingulate gyrus in MDD than in controls (Figure 7F).

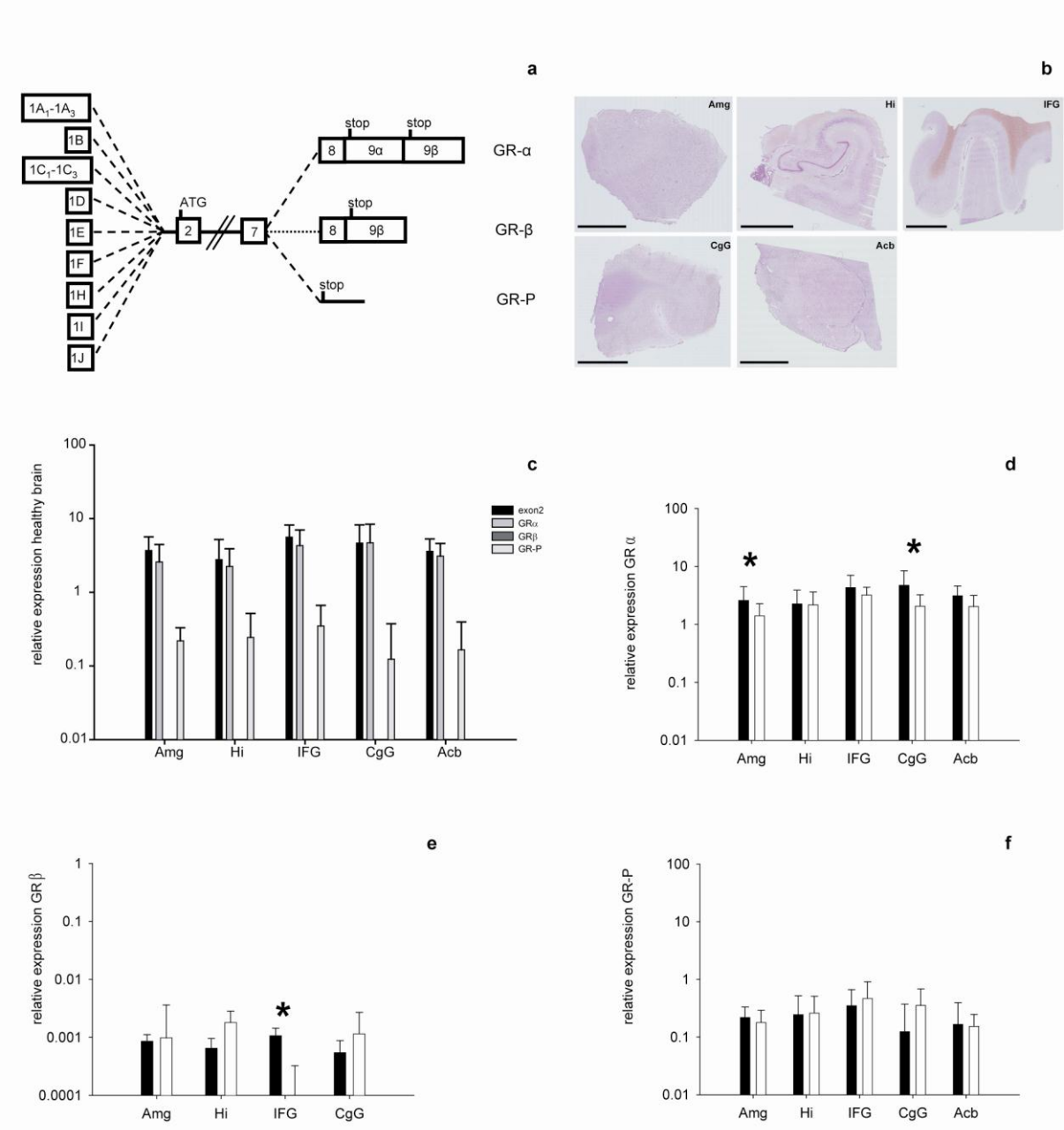

Figure 7:

The *GR* and its major isoform variants in selected limbic brain regions.

Expression of the *GR* gene (a). The various 5'-end untranslated exons are spliced to the same splice acceptor site in exon 2. 3'-End alternative splicing results in mRNAs encoding three variants of the *GR* gene: *GRα, GRβ* and *GR-P*. Cresyl violet stained sections for histological confirmation of amygdala (Amg), hippocampus (Hi), inferior prefrontal gyrus (IFG), cingulate gyrus (CgG) and nucleus accumbens (Acb) are represented in panel b. Scale bars represent 5 mm. Relative mRNA expression of all *GR* isoforms (c) in different non-depressed brain areas and *GRα* (d), *GRβ* (e) and *GR-P* (f) in control subjects compared to

MDD patients. The values are given as $2^{-(\Delta Ct)}$ with the geometric means normalised to *β-actin*. Closed bars represent non-depressed donors; open bars represent patients with MDD. ***** P<0.05.

Expression of *GR* first exon transcripts

GR first exon transcripts are differentially expressed in the human brain with transcripts of 1C and 1B being the most abundant and transcripts of 1E and 1J the least abundant (Figure 8A). Significant differences (p<0.05) in *GR* first exon expression were observed in several specific brain regions of MDD patients in comparison to non-depressed brains. Exon 1B was significantly lower in the nucleus accumbens (p=0.046, Figure 8B). Also, exon 1C (p=0.006, Figure 8C) and 1F (p=0.045, Figure 8F) showed a significantly lower expression in MDD hippocampi. In contrast, exon 1D was up-regulated in the cingulate gyrus (p=0.015, Figure 8D) and exon 1J in the amygdala (p=0.045, Figure 8H) of MDD patients. Interestingly, expression of *GR-P* and exon 1B usage correlated negatively in MDD patients over all brain areas (Figure 9E). A negative correlation was also found between usage of promoter 1C and alternative splicing to *GR-P* in MDD brains (Figure 9F).

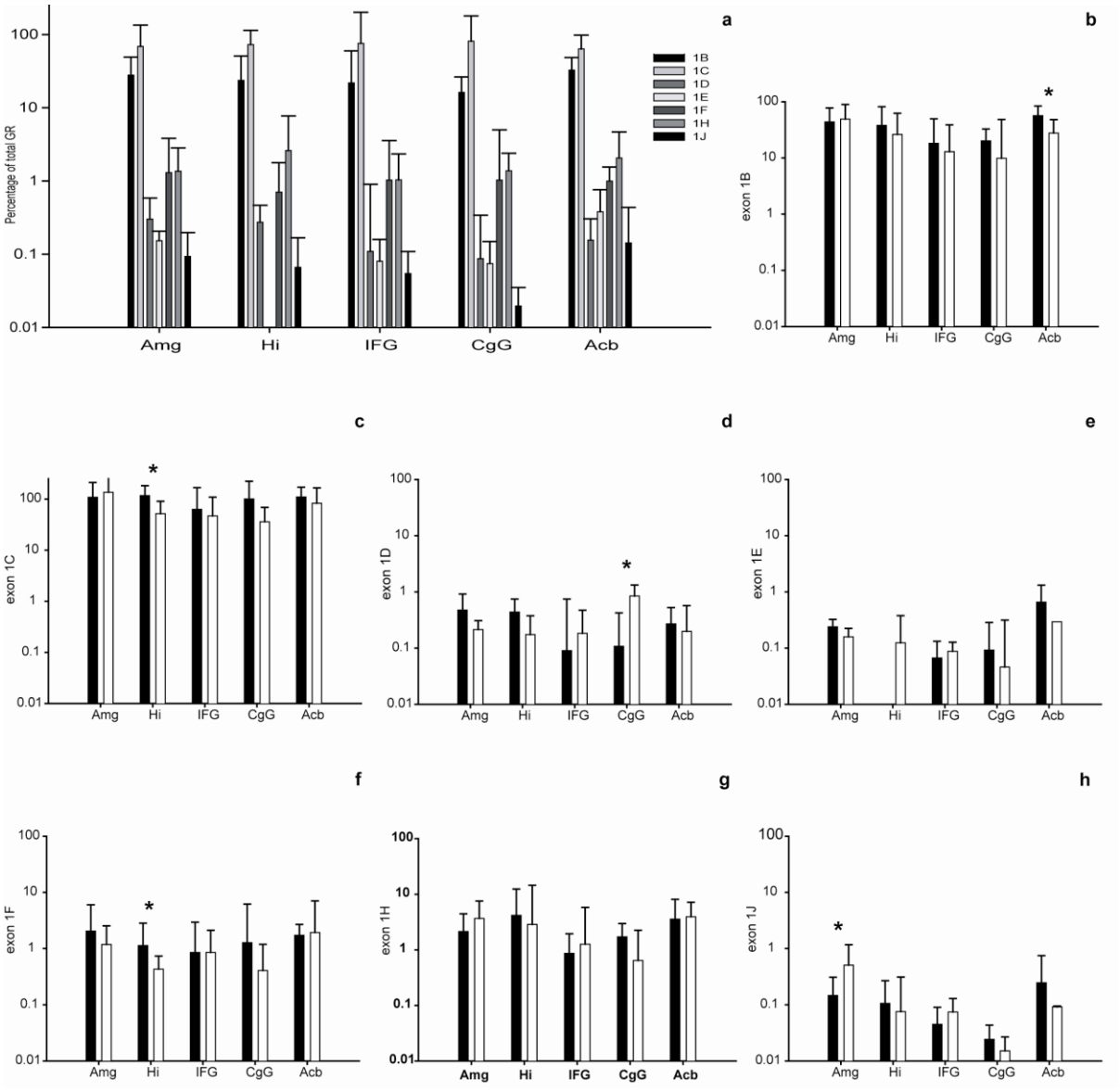

Figure 8:

GR first exon distribution in the non-depressed and depressed human brain.

Percentage of different *GR* first exons related to the total amount of *GR* in different non-depressed brain areas (a). Percentage of expression levels of *GR* first exons 1B (b), 1C (c), 1D (d), 1E (e), 1F (f), 1H (g) and 1J (h) in control subjects (closed bars) compared to MDD patients (open bars). The values are given as $2^{-(\Delta\Delta Ct)}$ with the geometric means normalised to *β-actin* and related to the total amount of *GR*. ***** P<0.05.

Expression of transcription factors regulating *GR* transcription

Interestingly, the transcription factor *YY1* was lower in the amygdala, the inferior prefrontal gyrus and the cingulate gyrus of MDD patients compared to controls (Figure 9A). Despite several YY1 binding sites upstream of exon 1D, no correlation was found between *YY1* and 1D expression in the amygdala of controls. The transcription factor *NGFI-A* was significantly decreased in the hippocampus, the cingulate gyrus and the nucleus accumbens (Figure 9B). *SP1,* another transcription factor known to regulate the GR, was significantly down-regulated in the inferior prefrontal gyrus and the cingulate gyrus of MDD patients (Figure 9C).

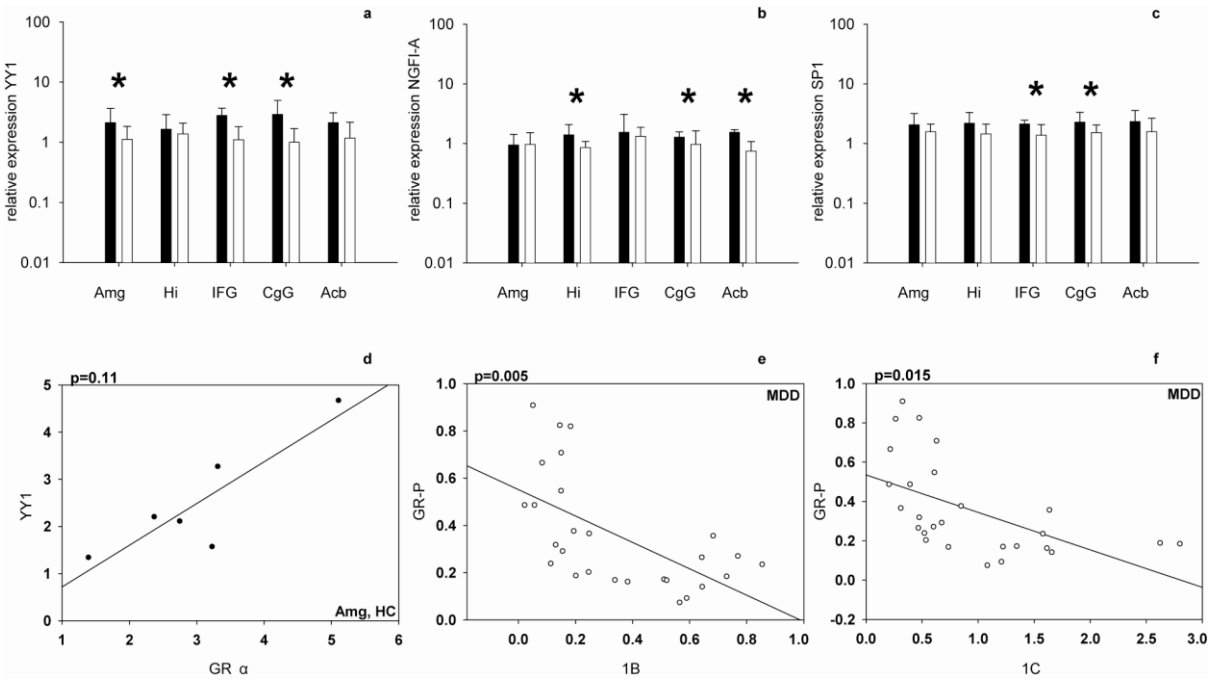

Figure 9:

Transcription factors regulating and internal splicing of, the *GR*.

Relative mRNA expression levels of *GR* regulating transcription factors *YY1* (a), *NGFI-A* (b) and *SP1* (c) in different brain areas. Closed bars represent non-depressed donors; open bars represent patients with MDD. Pearson correlations between mRNA expression levels of *GR* transcript variants, *GR* 3'-isoforms and transcription factors (d-f). Pearson's correlation co-efficient after Bonferroni correction, brain areas and donors are indicated in each panel. ***** P<0.05.

CpG methylation analysis

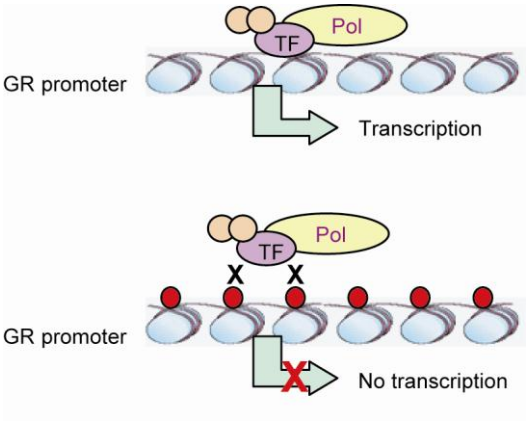

Figure 10:
DNA methylation inhibits transcriptional regulation of genes. Under normal conditions, a complex containing transcription factors (TF), the DNA polymerase (Pol) and co-activators (open circles) binds to the unmethylated promoter and regulates *GR* expression. If the transcription factor binding site in the promoter is methylated (full ovals), binding is inhibited and thus, *GR* expression is reduced.

Since methylation has a major impact on gene transcription (Figure 10) we pyrosequenced *GR* promoter regions 1J, 1E, 1B and 1F (Figure 11) and found very low overall levels of DNA methylation. 294bp were sequenced covering exon 1 J, part of its promoter as well as promoter 1E, including 13 CpG dinucleotides (Figure 11). Within this region a Pax3 binding site was located in promoter 1J (CpG [16]) and a predicted binding sites for CoupTF (CpG [17, 18] in promoter 1J), NFκB (CpG [24] in promoter 1E) and NGFI-A (CpG [27] in promoter 1E) (Supplement 2). No methylation was observed in most CpG sites neither in the control group nor the MDD group. However, in promoter 1E a low (6-9%) level of methylation was observed for CpG [24], within a NFκB binding site, and a moderate (29-33%) level of methylation was found in CpG [28]. No significant difference in methylation patterns was found between both groups and between different brain regions for any of the CpG sites within the above 294bp.

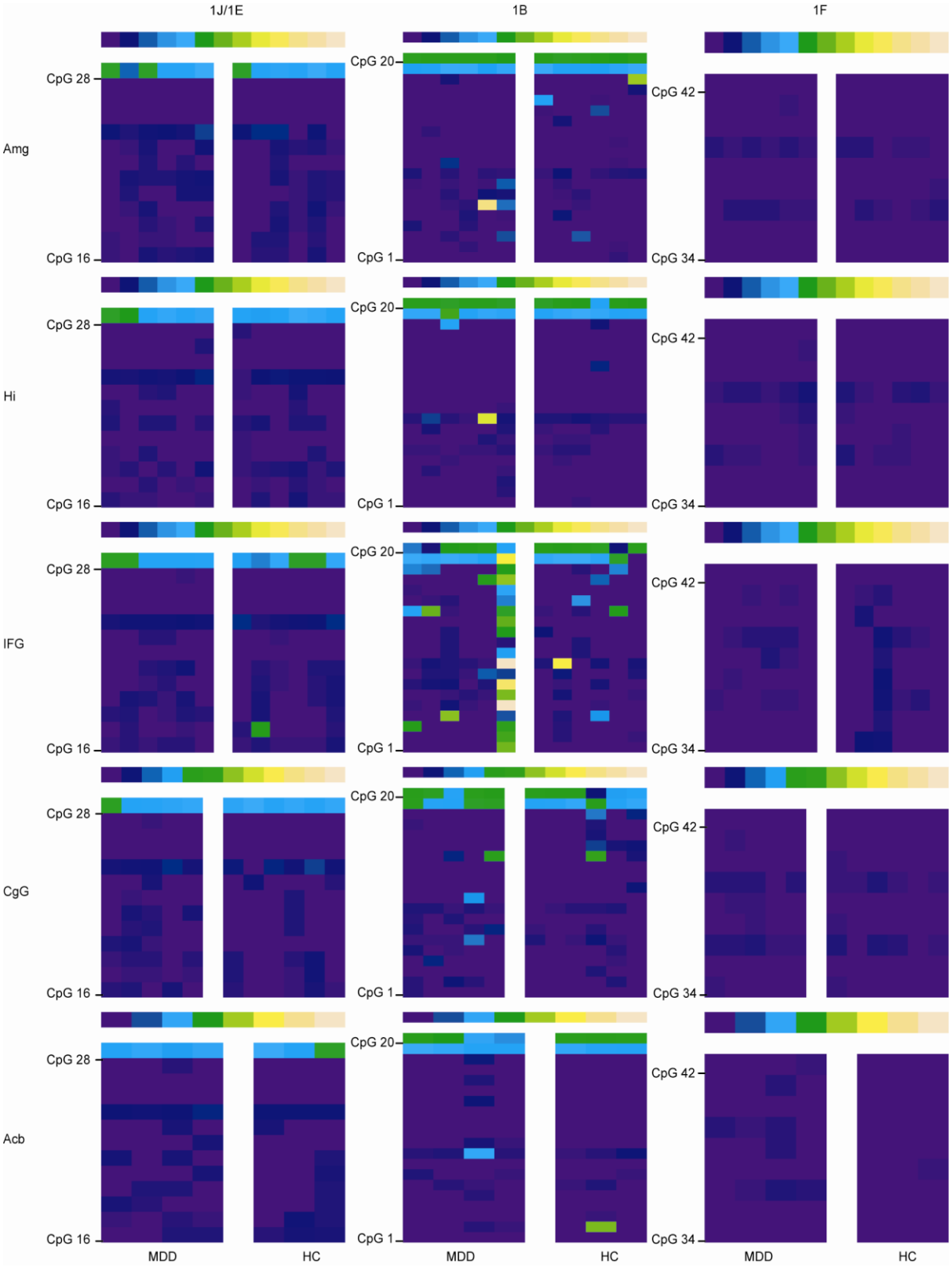
1J/1E
1B
1F
Amg
Hi
IFG
CgG
Acb
CpG 28
CpG 16
CpG 20
CpG 1
CpG 42
CpG 34
MDD
HC

Figure 11:

The *GR* promoter region is sparsely methylated in the human brain.

Percentage of methylation was measured by pyrosequencing in 6 patients with MDD and 6 non-depressed donors, covering promoter 1J, promoter 1E, promoter 1B and part of promoter 1F. Methylation levels are expressed by colour (0% (blue) to 100% (yellow)), levels from the scale at the panel top.

The methylation levels observed were low; however, individuals within groups were highly variable in both patterns and levels. In addition, 20 CpG sites were sequenced within promoter 1B (CpG $^{1\text{-}17}$) and within a part of exon 1B (CpG $^{17\text{-}20}$) including binding sites for SP1 at CpG $^{2\text{-}3}$ and CpG $^{15\text{-}18}$ (Supplement 2). Except for CpG $^{18,\ 19}$ which are located in exon 1B, no methylation was detected in the promoter region neither in control subjects nor in MDD patients (Figure 11).

Since methylation of a Ngfi-a binding site in promoter 1_7 has been reported in the rat, 9CpG sites within promoter 1F have been sequenced including the known NGFI-A binding site located at CpG ^{41} and ^{42} (Supplement 2). All analysed CpG sites were unmethylated in both groups (Figure 11).

Discussion

In humans most peripheral organs show a pronounced differential usage of alternative untranslated first exons of the *GR* (Turner et al. 2006). Here we extended our earlier studies on tissue-specific expression of 5´variants of the *GR* to different regions of the human brain of control subjects and MDD patients. Surprisingly little variation was observed throughout the brain regions, although differential expression patterns have been seen throughout the human body (Turner and Muller 2005). Transcripts 1C and 1B were the most abundant ones and transcripts 1E and 1J showed the lowest expression (Figure 8A) with transcript 1F reaching a mean expression of about 1%. This pattern is similar to peripheral tissues like lung, salivary gland and cells of the immune system and in contrast to prostate and adipose tissue where exons 1B and 1C were conspicuously absent (Turner and Muller 2005). This suggests a similar usage of promoters and the

generation of similar transcript and receptor variants throughout the limbic system, including the prefrontal cortex, hippocampus and the amygdala which are all involved in the circuitry of the limbic system that mediates HPA feedback regulation by GC (Jacobson and Sapolsky 1991).

Total *GR* levels, measured over exon 2 or exons 3/4 (expressed in all *GR* species), remained stable whether analysed against *β-actin* or *GAPDH*. However, in all brain regions *GRα*, by far the most abundant *GR* isoform was reduced although significance was only reached in the amygdala and the cingulate gyrus (Figure 7D). This is in line with a previous study on *GRα* expression in major depression (Webster et al. 2002; Knable et al. 2004) where a GRα specific riboprobe showed reduced *GR* expression in the frontal cortex and the hippocampus. Thus, our results may represent a shift in splicing from one isoform to another isoform not detected here. Very low levels of *GRβ* were found in control and MDD brains. This is in line with previous studies (DeRijk et al. 2003), suggesting a limited functional role for GRβ in the human brain. Our observations confirm that the GRα/β ratio will be largely unchanged although the significant reduction of *GRα* expression may have a functional effect in MDD. Moreover, changes in *GRα* levels will affect the balance between GR and MR. The MR has been shown to be changed in MDD (Young et al. 2003; Wang et al. 2008). Although the functional role remains unclear an imbalance between GR and MR has been postulated to confer an enhanced vulnerability to disease (De Kloet et al. 1998).

Although levels and patterns of alternative first exon expression were very similar between different brain regions, our results show significant changes ($p<0.05$) in differential usage of *GR* first exons in MDD. The 5'-end of the mRNA influences both splicing of the primary transcript and translation of the mature mRNA. For instance, the use of exon 1A rather than 1B or 1C was associated with increased translation efficiency of GRα (Pedersen and Vedeckis 2003). Splicing at the 3'-end depends on the 5'-sequence and altered promoter usage influences the resulting 3'-*GR* isoform (Russcher et al. 2007). Our findings support this hypothesis showing a statistical link between first and last exon usage. Interestingly, we found a strong negative correlation in MDD patients between *GR-P* expression and promoter 1B usage. We also found a negative correlation in MDD between the usage of promoter 1C with alternative splicing to *GR-P* (Figure 9E-F). This suggests that usage of promoter 1B or 1C will not preferentially initiate expression of *GR-P* in MDD. The effects of GR-P *in vivo* are poorly understood,

but it has been suggested that GR-P enhances GC bioactivity (Hagendorf et al. 2005). In MDD, we suggest that GR-P only plays a minor role considering that expression levels do not differ between control subjects and MDD patients.

Previous studies also suggested a link between the binding of specific transcription factors and *GR* transcript expression (Shrivastava and Calame 1994; Nobukuni et al. 1995; Breslin and Vedeckis 1998). The transcription factor *YY1* as well as the *GRα* isoform are reduced in several brain regions of MDD patients. So far YY1 binding sites are known in promoter 1D, but 1D transcripts only count for 0.1-0.5% of the total *GR*. As *GRα* is significantly reduced concomitantly with *YY1*, this suggests that there may be additional YY1 binding sites in other *GR* promoters regulating *GRα* expression.

The human promoter 1F contains a binding site for the transcription factor NGFI-A. In this study, *NGFI-A* was down-regulated in the hippocampus of MDD patients but not in non-depressed controls. Concomitantly exon 1F was reduced in this brain region. However, the lower activity of the minor promoter 1F which accounts for only 1% of *GR* promoter activity in the human brain was not associated with a reduction of *GRα* or total *GR* in the hippocampus. Our observation of low 1F expression is in contrast with a recent report that 1F represents 40-60% of the total *GR* (McGowan et al. 2009). However, it fully concords with the previously reported activity of promoter 1F where it represented 5% of the most active CpG island promoter (Geng et al. 2008); in peripheral organs expression of 1F was also shown to be very low compared to promoters 1B or 1C (Turner and Muller 2005; Presul et al. 2007). Thus, the importance of changes in such a minor promoter is currently unclear.

Previous studies suggested that the activity of some transcription factors regulating *GR* transcription is modulated by DNA methylation of their binding sites in *GR* promoters (Holliday and Pugh 1975; Riggs 1975). DNA methylation prevents transcription factor binding in promoter sequences leading to transcriptional repression of the gene (Bird 2002). In the present study low methylation levels were found in both MDD and the control group except for promoter 1B. Methylation levels of single CpG sites were quantified by pyrosequencing, allowing significant improvement in reliability and sensitivity over previous techniques (Dejeux et al. 2009). Similar to earlier observations in PBMC's (Turner et al. 2008) methylation levels and patterns of the latter promoter were highly variable between individuals, especially in the inferior prefrontal gyrus and

the cingulate gyrus. These differences that were both stochastic and unique to each donor could be the result of epigenetic programming but this does not seem to be related to MDD. Promoter 1F was uniformly unmethylated throughout all regions studied for both MDD and control subjects. This is consistent with data from Moser *et al*. (Moser et al. 2007), which showed no methylation of the NGFI-A binding site in several degenerative brain diseases. In the absence of methylation the transcription factor can bind to the 1F promoter and activate *GR* expression. Thus, the changes in *GR* expression in patients with MDD are not caused by epigenetic programming of *GR* promoters.

In humans adverse early life events such as childhood abuse are important risk factors for depression during adult life (Weiss et al. 1999; Battle et al. 2004; Lu et al. 2008). McGowan *et al.* (McGowan et al. 2009) have recently shown that methylation within the NGFI-A binding site of promoter 1F was high in the hippocampus of suicide victims with childhood abuse. Suicide victims without childhood abuse showed no conspicuous methylation. As suicide and MDD are often co-morbid, this is in line with our findings that this promoter is unmethylated in MDD patients. On the other hand, the incidence of childhood abuse is highly variable in depressed patients, ranging from 4-12% (Danese et al. 2008), 13% (Mullen et al. 1993), 35% (Young et al. 1997) to 45% (Nemeroff et al. 2003). Thus we cannot exclude that some of our MDD patients may have been victims of such childhood abuse events even if this was not reported and documented. If childhood abuse played an important role in our patients, our methylation results would be in conflict with the methylation data by McGowan *et al*. (McGowan et al. 2009), since we observed no CpG methylation in promoter 1F. The absence of methylation may be taken as an indication that our donors were not victims of abuse.

McGowan *et al.* further showed that methylation of promoter 1F reduces *NGFI-A* activity and promoter 1F transcripts. In our study we find that also in MDD patients 1F transcripts are reduced in hippocampus but not because of epigenetic promoter methylation, but due to lower expression of *NGFI-A.* While in the abused suicide victims total *GR* expression was reduced, we did not find a similar reduction of *GR* in the hippocampus of MDD patients. Reduction of total *GR* in the suicide victims may be because of additional methylation in other *GR* promoters and indicate that the minor promoter 1F plays a disproportional role in childhood abuse victims.

Any post-mortem study of depressed brain material will be limited in that most MDD patients have used antidepressant drugs. This was also the case in our study where 3 patients were treated with tricyclic antidepressants (TCA) and 2 others with selective serotonin reuptake inhibitors (SSRI). In rodents, *Ngfi-a* expression (Bjartmar et al. 2000) and *Gr* exon 1_7 expression (Yau et al. 2004) seem to be increased by SSRIs while TCAs were reported to have either no effect (Yau et al. 2004) or to up-regulate total *Gr* levels (Heydendael and Jacobson 2008). Nevertheless, *NGFI-A* and exon 1F were significantly down-regulated in our MDD patients. On the other hand, we cannot exclude that *GR* expression may have been influenced by TCA treatment despite differences between species and drugs.

A weakness of our study, as for many others on human post-mortem brain tissue, is the limited sample size and some of the statistical significance was lost after Bonferroni correction. The appropriate statistical test for such a small group is the Mann-Whitney U-test rather than a multivariate analysis such as an ANOVA test (Siegel and Castellan 1988). While a larger cohort would have a higher statistical power, this may be at the expense of less severe inclusion/ exclusion criteria and a more heterogeneous set of samples.

Although MDD patients without antidepressant treatment will be difficult to find, the results of our study may be considered partially preliminary until a study with a larger number of MDD patients who were positively documented to have no history of childhood abuse.

Nevertheless, the present study is the first to give an overview of *GR* transcript expression in multiple areas of the limbic system in humans. Even if distribution patterns throughout all analysed brain regions were similar we observed significant differences in 5'- and 3'-transcripts between control subjects and MDD patients. Our results show that changes in expression levels are not due to epigenetic methylation of *GR* promoters. These data suggest that there are two underlying pathomechanisms both resulting in lower exon 1F expression and in associated depressive symptoms. Early life adverse events programme susceptibility to depression by inhibition of 1F expression through promoter methylation. However, in MDD in the absence of childhood abuse, we show that promoter 1F usage is also impaired but because of a dearth of *NGFI-A*. These two similar, but nevertheless distinct, pathological mechanisms may help in explaining the

clinical similarity of MDD in the presence and absence of childhood abuse, as they both result in lower exon 1F levels. This also explains that susceptibility to depression can be either predisposed by early life adverse events or developed independent of such a condition. These parallel mechanisms towards a similar endpoint implicated in depression may of course also be relevant to other genes involved in the pathogenesis of depression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen.2009.09.001.

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Chapter 3

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

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Abstract

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). 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 six MDD patients and six non-depressed subjects. 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 analysed, 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. 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 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. 2007; 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 normalised 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 five human postmortem brain regions isolated from six MDD patients and six 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).

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 five 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 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 4 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 4: 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). 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).

	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 insufŽciency
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 insufŽciency
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)	-	-	-

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 synthesised 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 synthesised 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 synthesised 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 MasterPLUS 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 6 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.

Normalisation 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 two duplicate measurements was used for further calculations. For each tissue sample two cDNA samples were synthesised 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 normalisation, 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. Normalisation 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)= .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 five brain regions from six non-depressed subjects and calculated relative to the total *MR* levels in the hippocampus (Figure 12). 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 five 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*).

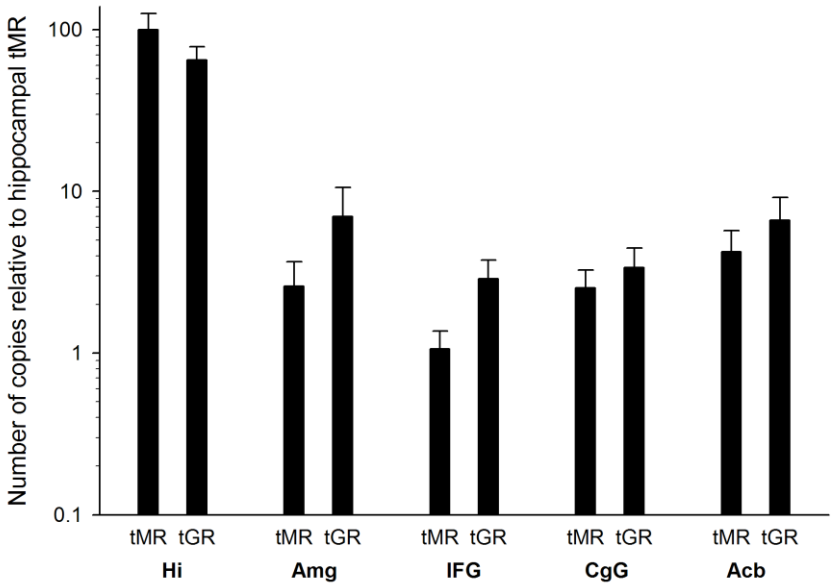

Figure 12:

Mean relative number of copies of total *MR* (tMR) and total *GR* (tGR) transcript compared between five brain regions of six 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 normalisation 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 five brain regions from six non-depressed subjects. Figure 13 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.

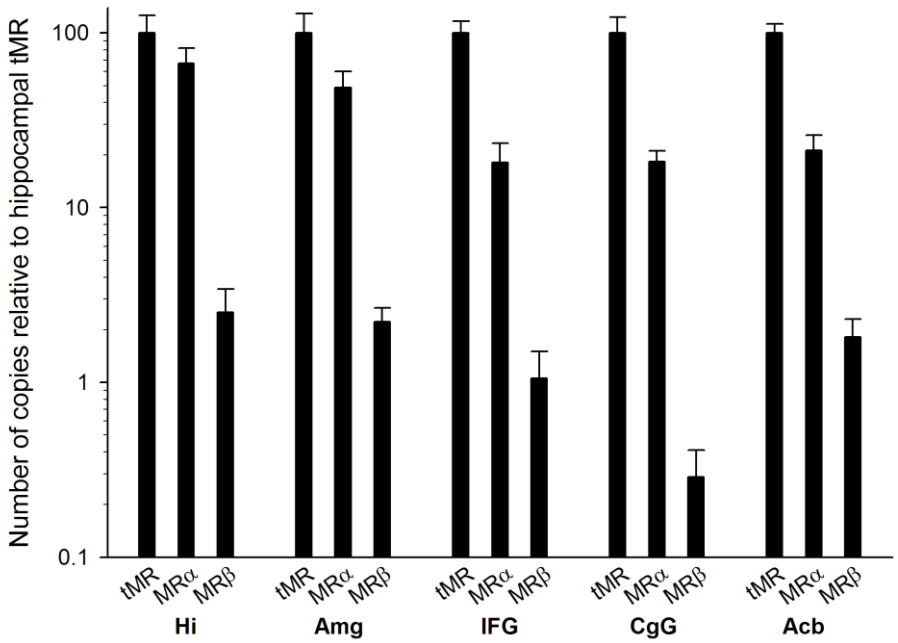

Figure 13:

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

MR and *GR* mRNA expression in depressed brain versus non-depressed brain

MR and *GR* transcript levels in five brain regions from six depressed subjects were determined and calculated relative to six non-depressed subjects (Figure 14). Differences in *MR* and *GR* expression were found in the hippocampus, inferior frontal gyrus and cingulate gyrus, while no differences were found in the amygdala or nucleus accumbens. Compared to the non-depressed brain, total *MR* mRNA expression was significantly lower (30%) in the hippocampus (p= .002) and cingulate gyrus (p= .03) of the depressed subjects. Total *GR* expression was significantly lower (30%) in the inferior frontal gyrus (p= .009) and a trend was found for the cingulate gyrus (p= .052). With respect to the *MR* splice variants, a significant lower expression of *MRα* was found in the cingulate gyrus (45%, p= .03), while the decrease was close to significance in hippocampus (30%, p= .07) respectively. Expression of *MRβ* was significantly lower in the hippocampus (40%, p= .03), inferior frontal gyrus (50%, p= .01) and cingulate gyrus (45%, p= .03), however, in the inferior frontal gyrus this did not result in a significant difference in total *MR* mRNA (p= .09). 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.

MR/GR ratio in brain of depressed compared to non-depressed subjects

For the six depressed subjects the total *MR/GR* ratio was calculated in five brain regions and compared with the six 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.

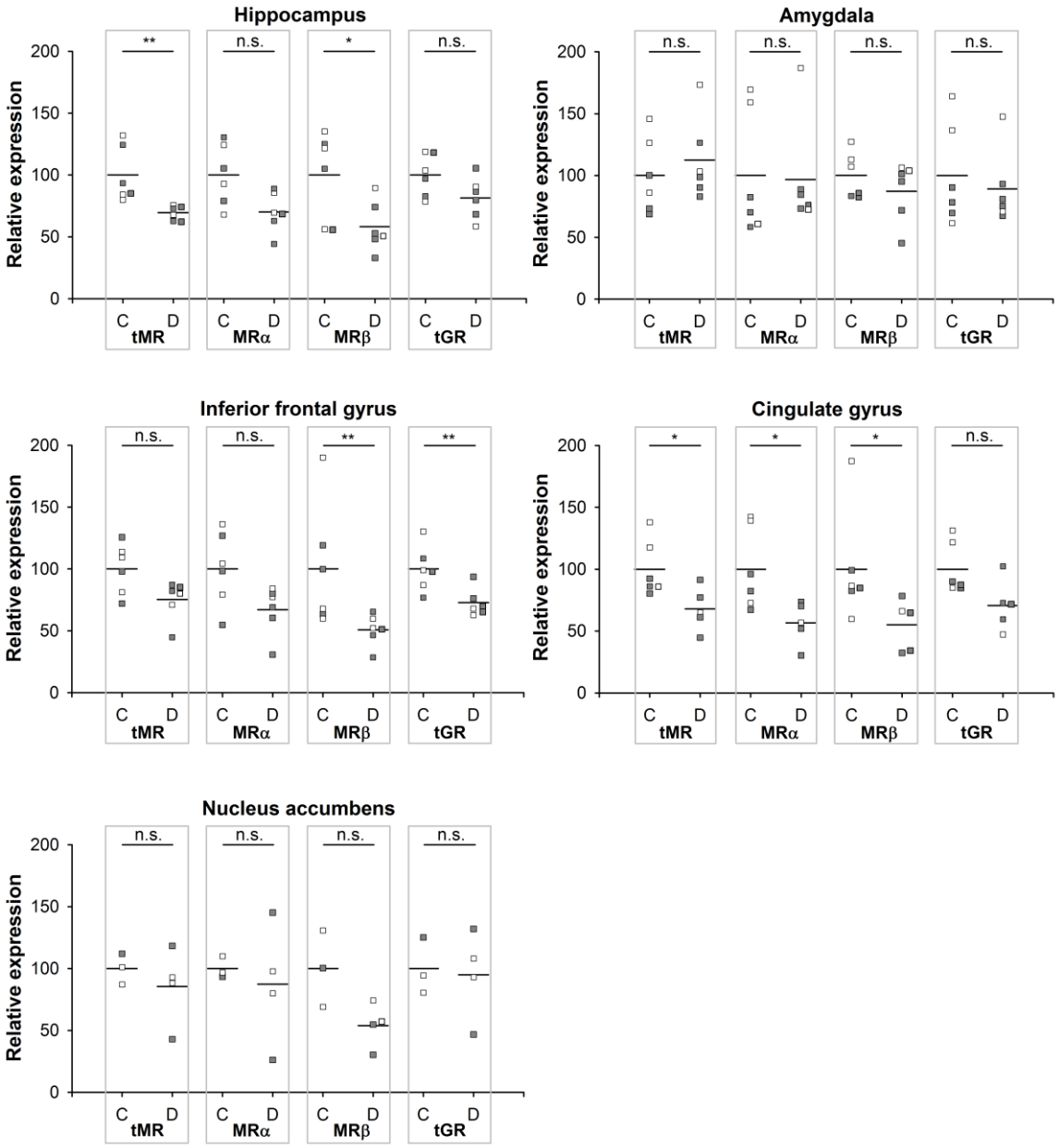

Figure 14:

Mean expression of total *MR* (tMR), *MRα*, *MRβ* or total *GR* (tGR) mRNA levels in five 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 normalisation with *tubulin*. Note that the expression levels are plotted on a linear scale. * $p < .05$; ** $p < .01$; n.s.= not significant.

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, *MR*α and *MR*β, were detected in all five brain regions, although expression levels of *MR*β were around 10 to 60 times lower than for *MR*α 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 *Mr*α versus *Mr*β ratio in the rat hippocampus varies throughout development (Vazquez et al. 1998), with *Mr*β being predominantly expressed during the second week after birth and showing a subsequent decrease up into the adult age. At adult age *Mr*β expression seems to be lower than *Mr*α expression. In the present study *MR*β expression levels were much lower compared to *MR*α. As all 12 subjects were of middle age or older (mean age 71.8 ± 13.9) this may suggest that central *MR*β expression decreases even further during aging. The levels of expression of *MR*α together with *MR*β 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, exon1γ (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 sample no significant group difference in total *GR* expression was reported for any of the five 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, *MR*β 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 previously 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. 2010; van Leeuwen et al. 2011); Klok et al., unpublished data). 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 for psychopathology (DeRijk et al. 2006; Kuningas et al. 2007; van Leeuwen et al. 2010; Klok et al. 2011); Klok et al., unpublished data). 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 normalised 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. 2007; 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 i*s 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 brain 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.

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Chapter 4

Transcriptional regulation of the glucocorticoid receptor transcript 1F: NGFI-A and E2F1?

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Abstract

The human *GR* promoter 1F is susceptible to methylation during stressful early life events resulting in lower 1F transcript levels. It was proposed that this is due to methylation of a hypothetical NGFI-A binding site in this promoter. In this study, the role of NGFI-A in regulating 1F transcription was analysed using transient transfections, reporter gene assays and chromatin immunoprecipitation in cells representing peripheral (293FT) and central (U373MG) tissue. Chromatin immunoprecipitation confirmed NGFI-A binding to the *GR* 1F promoter. NGFI-A induced 1F transcripts in the reporter gene assay, but had no effect on endogenous transcript levels. Several other transcription factors predicted by *in silico* phylogenetic footprinting to bind the 1F promoter were analysed. E2F1 was identified to bind to and to strongly up-regulate the expression of the 1F reporter gene. The E2F1 binding site in promoter 1F also covers a CpG site highly methylated in association with childhood abuse. Together with our data this suggests an important role for E2F1 in *GR* 1F regulation. Here, for the first time, we provide evidence that E2F1 is a major element in the transcriptional complex capable of driving the expression of *GR* 1F transcripts.

Keywords

Alternative first exons, Behavioural epigenetics, Chromatin immunoprecipitation, Glucocorticoid receptor, Stress response, Transcription factor

Introduction

The human glucocorticoid receptor (GR) is an ubiquitously expressed steroid hormone receptor with a pivotal role in maintaining homeostasis under stress. The 5'-region of the *GR* gene (OMIM + 138040; *NR3C1*) consists of 9 alternative first exons designated exons 1A to 1I in humans, reviewed in (Turner et al. 2010). This 5'-heterogeneity remains untranslated as the ATG start codon lies within exon 2, but it orchestrates translational regulation (Pickering and Willis 2005). Glucocorticoids are the end product of the hypothalamic-pituitary-adrenal (HPA) stress axis. In the rat, the HPA axis feedback sensitivity is thought to be dependent upon expression levels of *GR* first exons, which in turn have been shown to be susceptible to environmental influences such as early life stress in both rodents and man (Weaver et al. 2004; McGowan et al. 2009). Rodents experiencing poor maternal care, measured by licking/grooming and arched-back nursing (LG-ABN), had higher methylation levels throughout promoter 1_7 with the first CpG dinucleotide in the Ngfi-a binding site (also known as Egr-1; Zif-268; Krox-24) being especially susceptible to methylation or demethylation (Weaver et al. 2004). In humans, negative early life experience induced methylation at another, hypothetical, NGFI-A binding site (CpG 32) in the 1F promoter (McGowan et al. 2009). However, alterations in *GR* levels are not exclusively due to promoter methylation. We previously showed that, although methylation levels were highly variable in peripheral blood mononuclear cells (PBMC's) between individuals, the 2 key CpG sites (37-38) of the promoter 1F NGFI-A binding site were methylated at 10% or less (Turner *et al.* 2008). In the normal brain or the brain of patients with major depressive disorder (MDD), we and others detected at maximum 4% methylation in the 1F promoter, and only 0-2% in the NGFIA binding site (Moser et al. 2007; Alt et al. 2010). However, we showed decreased levels of 1F and *NGFI-A* expression in the hippocampus of MDD patients, suggesting a link between *NGFI-A* and *GR* 1F expression, although without measurable promoter methylation. In this present study, we further investigated the role of NGFI-A in the transcriptional regulation of the *GR in vitro*. We confirmed that NGFI-A binds to the 1F promoter, activates the 1F reporter gene but does not induce endogenous 1F transcripts. As NGFI-A seems to be unable to directly affect 1F transcription we tested several other transcription factors predicted to bind the 1F promoter. We found the transcription factors ELK1 and especially E2F1 to strongly up-regulate 1F promoter activity. Therefore, we suggest that E2F1 is required for driving the expression of the *GR* 1F transcripts.

Materials and Methods

Cell cultures

Human embryonic kidney (293FT) cells (Invitrogen) were cultured at 37°C and 5% CO_2 in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum, 1% non essential aminoacids, 1% Ultraglutamine and 1% sodium pyruvate (Gibco-BRL, Breda, The Netherlands). Human glioblastoma-astrocytoma (U373MG) cells (Prof. Jobst Meyer, University of Trier, Germany) were cultured in EMEM medium (Lonza) containing 20% fetal bovine serum.

GFP expression vectors and luciferase reporter gene assay

The full length *NGFI-A* coding sequence was amplified from pCMV6-XL5-EGR1 (Origene, Rockville, MD, USA), all other transcription factor coding sequences were subcloned from specific pCMV-SPORT6 clones (ImaGenes, Berlin, Germany) as described by Cao-Lei *et al.,* 2011. The human *GR* promoter 1F (-3536/-3211) luciferase construct was generated and reporter gene assays were performed as described previously (Cao-Lei et al. 2011). PCR primer sequences, restriction enzymes, annealing temperatures and $MgCl_2$ concentrations are shown in Table 5.

Preparation of cDNA in 293FT and U373MG cells and real-time qPCR

Messenger RNA was purified 48h post-transfection using a µMACS™ mRNA Isolation Kit (Miltenyi Biotech, Utrecht, The Netherlands). RNA quantity was measured on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). First-strand synthesis of total cDNA and real time PCR were carried out as previously described (Alt et al. 2010). Primers and PCR conditions are shown in Table 5.

Table 5: Primer sequences and PCR conditions.

expression vector constructs	Primer sequences	Tm (°C)	MgCl2 (mM)	Primers (uM)	Product (bp)	DNA Polymerase
pNGFI-A-Ac-GFP	fwd: 5'-GAACCGTCAGATCCGCTAGCATGGCCGCGGCCAAGGCCGAGAT-3' rev: 5'-GCCCGCGGTACC GTCGAC TTAGCAAATTTCAATTGTCCTG-3'	64	2	0.5	1587	Pl-TP
pE2F1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCCGTGAGCGTCATGGCCTTGG-3' rev: 5'-GTCGACTGCAGAATTCATCTCTGGAAACCCTGGTCCCTC-3'	66	0.5	0.3;0.5;0.7	1388	Ph-TP
pRFX1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCTCATTTTCCCCACCGTTGGCATGG-3' rev: 5'-GTCGACTGCAGAATTCACCCTGGCGTGGAGGGGTGGC-3'	64	1.5	0.3;0.5;0.7	3043	Pl-TP
pELK1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCAACAGTACCCCTGGGATGGC-3' rev: 5'-GAAGCTTGAGCTCGAGCCAGAAGGGGTGGTGGTGGT-3'	66	0.7	0.3;0.5;0.7	1388	Ph-TP
pEBF1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCGAGGAGATTTTCCACAAGAA-3' rev: 5'-GATCCCGGGCCCGCGGCCAACCTCTTCATTAATACA-3'	64	0.5	0.5;0.7	1879	Ph-TP
pHES1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCCGTGAAGAACTCCAAAAATA-3' rev: 5'-GTCGACTGCAGAATTCGTTGGGGAGTTTAGGAGGAG-3'	60	0.5	0.7	968	Ph-TP
pMetLuc-1-F	fwd: 5'-TACCGGACTCAGATCTAAGTACGTATGCGCCGACCC-3' rev: 5'- GTCTACTGCAGAATTC CTCGCTACCTCCTTCCCGCC-3'	67	0.5	1	326	Ph-TP
plasmid sequencing primers						
pIRES-AcGFP	fwd: 5'-ATAGTCCTGTCGGGTTTCGCC-3' rev: 5'- CTCTTGAAGAAGTCGTGCTGCT-3'					
pMetLuc-1-F	fwd: 5'- AACCGTATTACCGCCATGCA-3' rev: 5'- AACACCACCTTGATGTCCATGG-3'					
pE2F1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-GCTTCGGCCAGTAACGTTAG-3' fwd-2: 5'-ACTGAATCTGACCACCAAGC-3' rev-2: 5'-CGGTACATGCACACACACAT-3'					
pRFX1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-GCTTCGGCCAGTAACGTTAG-3' fwd-2: 5'-TGGTACCCAGCAGGTGCACT-3' rev-2: 5'-CACGGGAGGGGGCCTGCCTG-3' fwd-3: 5'-ACCCGCCGTCTGGGCACCAG-3' rev-3: 5'-CAGGCCGCCCACTGCTCCAG-3'					
pELK1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-AAGCTCTTCCGATTTCAGGT-3' fwd-2: 5'-CTGTCTGGAGGCTGAAGAGG-3' rev-2: 5'-GCTTCGGCCAGTAACGTTAG-3'					
pEBF1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-TTCTTGTGGAAAATCTCCTCC-3' fwd-2: 5'-GATCCAGTGATAATTGACAG-3' rev-2: 5'-ACACTGTACAGGGCCTCGGC-3' fwd-3: 5'-AAGCACTGTATGGGATGCCA-3' rev-3: 5'-GCTTCGGCCAGTAACGTTAG-3'					
pHES1-Ac-GFP	fwd: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev: 5'-GCTTCGGCCAGTAACGTTAG-3'					
expression analysis						
b-actin	fwd: 5'-GGCCACGGCTGCTTC-3' rev: 5'-GTTGGCGTACAGGTCTTTGC-3'	60	2	1	208	Pl-TP
GAPDH	fwd: 5'-GAAGGTGAAGGTCGGAGTC-3' rev: 5'-GAAGATGGTGATGGGATTTC-3'	60	2	1	228	Pl-TP
PPIA	fwd: 5'-TCCTGGCATCTTGTCCATG-3' rev: 5'-CCATCCAACCACTCAGTCTTG-3'	60	1	1	90	Pl-TP
NGFI-A	fwd: 5'-ACCGGCCCAGCAAGACGCC-3' rev: 5'-GGGCACAGGGGATGGGTATG-3'	60	2	0.5	437	Pl-TP
E2F1	fwd: 5'-TGAATATCTGTACTACGCAG-3' rev: 5'-AGATGATGGTGGTGGTGACA-3'	56	4	0.4	341	Pl-TP
GR 1F	fwd: 5'-GTAGCGAGAAAAGAAACTGG-3' rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	60	1.75	0.5	511	Pl-TP
exon 2	fwd: 5'-GAGGGGAGATGTGATGGACTTCT-3' rev: 5'-GCTGCTGCGCATTGCTTA-3'	55	2.5	0.8	170	Pl-TP
ChIP PCR						
1-F	fwd: 5'-TACCGGACTCAGATCTAAGTACGTATGCGCCGACCC-3' rev: 5'- GTCTACTGCAGAATTC CTCGCTACCTCCTTCCCGCC-3'	64	3	0.1	370	D-TP

Diamond Taq polymerase: D-TP
Platinum Taq polymerase: Pl-TP
Phusion Taq polymerase: Ph-TP

Protein extraction and Western blotting

Whole cell protein was extracted 48h post transfection with a cell lysis solution (pH 8.5) containing 7M urea, 2M thiourea, 4% CHAPS, 30mM Tris, 12% isopropanol, a protease inhibitor cocktail and a nuclease mix. All extractions were performed in triplicate from different cell passages. Protein concentrations were quantified with the 2D-quant kit (Amersham Biosciences, Diegem, Belgium). Proteins were resolved on a 4–16% Bis-Tris Novex gel (Invitrogen) and transferred to a nitrocellulose membrane (GE healthcare, Diegem, Belgium). Membranes were subsequently incubated with rabbit polyclonal hNGFI-A (sc-110), hE2F1 (sc-193) antibodies and a mouse monoclonal hβ-Actin (sc-47778) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and visualised with anti-rabbit Cy5 and anti-mouse Cy3 labelled secondary antibodies (Pierce, Rockford, IL) using a standard protocol (Towbin et al. 1979).

Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed in duplicate using the Low cell ChIP kit (Diagenode, Liege, Belgium). Briefly, $6x10^6$ U373MG or 293FT cells were crosslinked (1% formaldehyde), sheared (Bioruptor 200, Diagenode) and immunoprecipitated using rabbit polyclonal antibodies: NGFI-A (sc-110, Santa Cruz), E2F1 (sc-193, Santa Cruz), H3K9/14Ac, H3K4Me3 (both marker for active chromatin), H3K9Me3 and H3K27Me3 (marker for heterochromatin, all histone antibodies from Diagenode). Normal rabbit non-immune IgG (sc-2027, Santa Cruz) was used as a negative antibody. The *GR* exon 1F promoter region of the uncrosslinked DNA was subsequently amplified by PCR (conditions are shown in Table 5). PCR products were separated on a 2% agarose gel and visualised with SYBR Safe (Invitrogen) under UV light.

Statistical analysis

The gene specific threshold cycle (Ct) was used for semi-quantitative assessment of RT-PCR data using a stable internal reference gene (*β-actin*). The amount of mRNA in the transfected cells relative to the one in the untransfected cells was calculated as $2^{-(\Delta Ct)}$ according to Livak and Schmittgen (Livak and Schmittgen 2001). PCR

efficiencies showing high linearity (R^2 > 0.993) after 7-log dilution series varied between E = 1.85 – 2.15.

All results were analysed using a one way analysis of variance (ANOVA) followed by Tukey post-hoc correction. Data from controls were compared to those of stressed animals using a Kruskal–Wallis test followed by a Dunn's procedure for post-hoc correction. Statistical analyses were performed using Sigma Stat for Windows (Erkrath, Germany). Differences were considered significant when $p<0.05$ after post-hoc correction.

Results

Overexpression of *NGFI-A* and 1F transcript levels

After transient transfection, real-time PCR revealed that a 1500 fold (p=0.012) or even 1900 fold (p=0.009) overexpression of *NGFI-A* had no effect on the level of endogenous exon 1F or total *GR* expression in 293FT or U373MG cells (Figure 15A/B). However, co-transfection of the NGFI-A expression vector with the 1F reporter gene showed a 2.5 fold induction in 1F promoter activity in 293FT cells but not in U373MG cells (Figure 16A/B). Thus, NGFI-A is either a week inducer of the native 1F transcript or additional transcription factors are necessary for its activation. Overexpression of NGFI-A (Figure 15A/B) was confirmed by Western Blot analysis (Figure 16C).

NGFI-A binding to the human *GR* promoter 1F

To confirm that NGFI-A binds to the *GR* promoter 1F, we performed chromatin immunoprecipitation in 293FT and U373MG cells. As the chromatin structure can block the promoters accessibility, the well known active chromatin markers acetyl-lysine-9 (K9) residues of histones H3 and H4 (Kadonaga 1998) were used as positive controls. Successful amplification of promoter 1F confirmed the euchromatin

character of promoter 1F and its accessibility for transcription factor binding (Figure 16D). After NGFI-A specific immunoprecipitation, the promoter 1F was amplified from genomic DNA demonstrating that NGFI-A was bound to promoter 1F. Immunoprecipitation of the NGFI-A promoter 1F complex was inhibited by pre-incubation of the antibody with its blocking peptide, confirming the specificity of the IP.

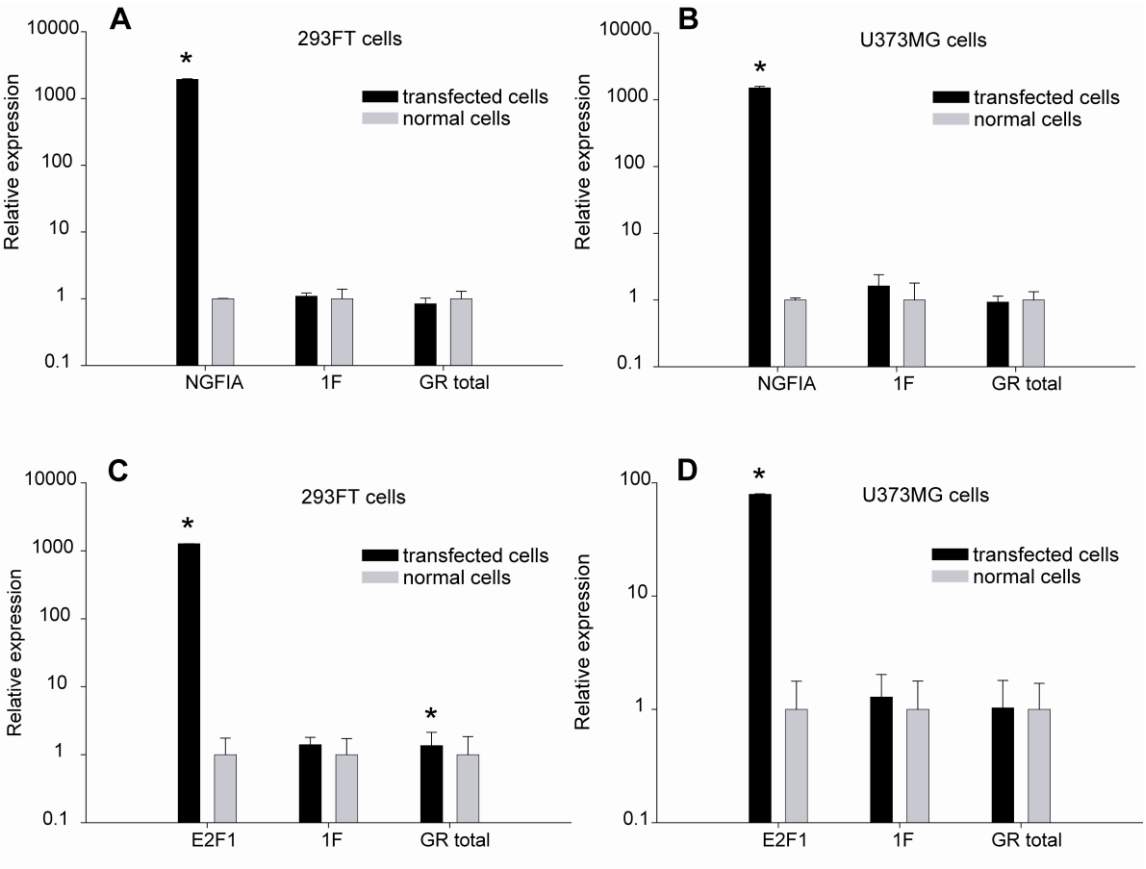

Figure 15:

The effect of NGFI-A and E2F1 on human 1F transcript expression.

Relative mRNA expression of *NGFI-A*, exon 1F and total *GR* in 293FT (A) and U373MG (B) cells after transfection with a NGFI-A expression vector was measured by real-time PCR. Relative mRNA expression of *E2F1*, exon 1F and total *GR* in 293FT (C) and U373MG (D) cells after transfection with an E2F1 expression vector was measured by real-time PCR. The values are given as $2^{-(\Delta Ct)}$ with the geometric means normalised to *β-actin*. * P<0.05.

Effects of different transcription factors on the promoter 1F reporter gene

Computational methods (Turner et al. 2008) previously predicted several transcription factor binding sites evolutionary conserved in promoter 1F of eukaryotes. The strongest of these predictions (RFX1, E2F1, ELK1, EBF1 and HES1) were cloned into the pIRES-AcGFP vector and individually co-transfected with the promoter 1F reporter gene. Successful overexpression of the different transcription factors was confirmed by Western Blot analysis (Figure 16C). In 293FT cells NGFI-A, E2F1 and ELK1 lead to an increase in luciferase activity (Figure 16A). E2F1 increased 1F transcription almost 6 fold while the overexpression of ELK1 and NGFI-A resulted in a 2.5 fold increase of 1F promoter activity. In U373MG cells the overexpression of NGFI-A did not up-regulate 1F activity (Figure 16B). ELK1 increased 1F promoter activity by 2.5 fold, as in 293FT cells. E2F1 overexpression increased the 1F promoter activity ~4 fold. All other transcription factors analysed had no significant effect on reporter gene activity.

E2F1 binds to the endogenous 1F promoter

Since E2F1 induced the 1F promoter in the reporter gene assays, we also evaluated its effect on endogenous 1F levels in 293FT and U373MG cells. Although E2F1 was strongly overexpressed ($p<0.001$ in 293FT cells; $p=0.019$ in U373MG cells) as confirmed by Western Blot analysis (Figure 16C), this did not increase 1F transcripts in 293FT cells (Figure 15C) nor in U373MG cells (Figure 15D). Although E2F1 was not able to increase endogenous 1F expression levels, chromatin immunoprecipitation with an E2F1 antibody showed that in both cell lines E2F1 binds to the endogenous 1F promoter (Figure 16D).

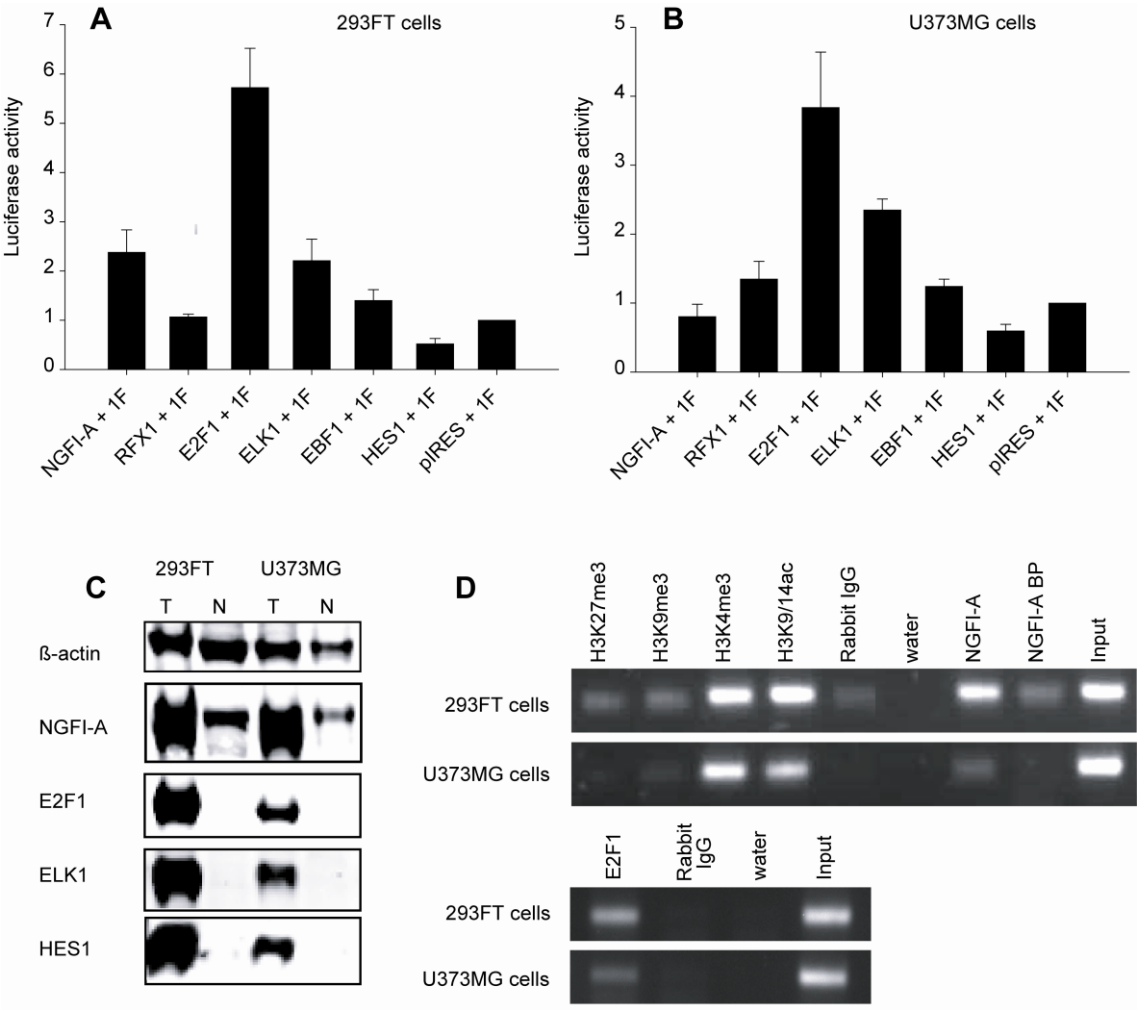

Figure 16:

Induction of 1F promoter activity by other predicted transcription factors.

Luciferase activity (mean ± SD) of the 1F reporter gene co-transfected with different transcription factor expression vectors and normalised to the co-transfection with the empty expression vector (pIRES-1F) in 293FT (A) and U373MG cells (B). Western blot analysis of different transcription factors in transfected (T) and untransfected (N) cells to confirm overexpression (C).

Chromatin immunoprecipitation (D) of genomic DNA from 293FT as well as U373MG cells using different histone antibodies as markers for inactive (H3K27me3, H3K9me3) or active (H3K4me3, H3K9/14ac) chromatin. Precipitation with a NGFI-A specific antibody (sc-110) was followed by amplification of the 1F promoter region (370bp). Specificity of the antibody was checked by using a blocking peptide (NGFI-A BP) preventing binding of the antibody to the DNA. Chromatin immunoprecipitation with an E2F1 specific antibody (sc-193) was followed by amplification of the 1F promoter region. Normal rabbit IgG and water were used as negative controls whereas the Input was used as a positive control for amplification. * $P<0.05$.

Discussion

It has been shown previously that NGFI-A is involved in the transcriptional regulation of the promoter of the human alternative exon 1F and its rat homologue 1_7 and that both are subject to epigenetic regulation (Weaver et al. 2004; McGowan et al. 2009). However, we and others have observed a poor correlation between promoter methylation and altered 1F expression levels (Moser et al. 2007; Oberlander et al. 2008; Alt et al. 2010) suggesting that other mechanisms play a role, especially since promoters are rarely dependent on a single transcription factor (reviewed in Turner et al., 2010).

Using chromatin immunoprecipitation, we confirmed that the DNA is accessible for transcriptional regulation and that NGFI-A binds to the human promoter 1F, at least in the two cell types tested. Previous studies reported a 4 fold increase in the 1F promoter activity (McGowan et al. 2009) and in the rat 1_7 (Weaver et al. 2007) when reporter genes were co-transfected with the human and the rat NGFI-A, respectively. However, in neither of these studies, the natural 1_7 or 1F transcripts of the cells were measured. We showed that NGFI-A overexpression does not induce the endogenous 1F transcript. Thus, our data suggests that NGFI-A may be part of the transcriptional complex that activates the reporter gene, but that this is not sufficient to activate the natural 1F promoter *in vitro*.

Analysis of the human *GR* 1F promoter suggested that NGFI-A is only one of a series of transcription factors that regulates the expression of the 1F transcript. In our reporter gene system ELK1 had a similar or even higher activation capacity compared to NGFI-A. The transcription factor E2F1, one of our most robust predictions, showed a significantly higher transcriptional activity than NGFI-A in both cell lines. E2F1 overexpression significantly increased promoter 1F both in 293FT cells (6 fold) and U373MG cells (4 fold) in our reporter gene system. Moreover, we demonstrated binding of E2F1 to promoter 1F by immunoprecipitation. In these cell lines there is no evidence for regulation of the endogenous gene, however, the evidence for the two additional transcription factors being regulators of GR expression is stronger than that for NGFI-A.

The E2F1 binding site predicted by *in silico* phylogenetic footprinting (Turner et al. 2008) covers CpG position 32 that, in human suicide victims with a history of childhood abuse, was highly methylated (40%) in comparison to suicide victims without childhood abuse (15%) (McGowan et al. 2009). The post-mortem samples

from the abused victims had lower 1F transcript levels in agreement with their higher methylation levels. The authors suggested that CpG 32 was part of a second, hypothetical NGFI-A binding site. We suggest that E2F1 binds in this region and that reduced E2F1 binding as a result of DNA methylation could be responsible for the lower 1F expression observed previously (McGowan et al. 2009).

In conclusion, the region immediately up-stream of exon 1F is the target of multiple transcription factors. We did not observe a dominant effect of NGFI-A in our reporter gene system and both ELK1 and E2F1 were more effective. Using chromatin immunoprecipitation, we demonstrated that the promoter 1F chromatin structure is compatible with transcription and that both NGFI-A and E2F1 bind to the endogenous *GR* 1F promoter. Therefore, we suggest E2F1 as a major element in the complex necessary to control *GR* 1F transcription *in vitro* and *in vivo*.

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Chapter 5

Dichotomy in methylation patterns and sensitivity to acute stress of glucocorticoid receptor promoter 1_7 in the hypothalamus and the hippocampus

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Submitted.

Summary

Expression levels of the glucocorticoid receptor (Gr) in the brain are an important determinant of stress responsiveness. The *Gr* is regulated by multiple alternative first exons each with its own promoter. It has been suggested that methylation of an Ngfi-a response element regulates the activity of the *Gr* 1_7 promoter in the rat brain. Here, we investigated the impact of restraint stress on the expression of *Ngfi-a* and *Gr* 1_7, and on methylation levels of the 1_7 promoter. Restraint stress induced *Ngfi-a* in the paraventricular nucleus of the hypothalamus (PVN) and cortex, but this had no influence on 1_7 transcript levels despite low average methylation levels throughout the 1_7 promoter. Pyrosequencing of the 1_7 promoter in the PVN revealed a striking variability in methylation of some of the CpG sites (0-68%) and *Gr* 1_7 expression levels correlated negatively with methylation. In contrast, methylation levels in the hippocampus were less variable and on average below 15%. However, a significant increase in methylation was observed at 4 CpG sites in the hippocampus four hours after acute stress demonstrating some plasticity of methylation patterns even during adulthood. Our results suggest that the rat promoter 1_7 was not transcriptionally regulated by Ngfi-a and revealed a striking dichotomy in the PVN and the hippocampus in variability of methylation levels and susceptibility of the *Gr* promoter 1_7 to methylation under acute stress. These results suggest a remarkable differential 'state' versus 'trait' regulation of the *Gr* gene in different brain regions.

Keywords

epigenetics, glucocorticoid receptor, hippocampus, PVN, rats, stress, transcription factor

Introduction

Regulation of the expression of the glucocorticoid receptor (GR) in the brain is part of adaptation to stress (de Kloet et al. 2005). Inappropriate GR levels have been linked to psychopathology, for example in relation to epigenetic early life programming. The 5'-region of the *GR* gene (OMIM + 138040; *NR3C1*) consists in humans of 9 untranslated alternative first exons (1A to 1I) (Breslin et al. 2001; Turner and Muller 2005; Presul et al. 2007); *Gr* first exons are equally variable in the rat (11 first exons, 1_1 to 1_{11}) and 8 exons (1_4 to 1_{11}) are highly homologous to the human exons (McCormick et al. 2000; Turner and Muller 2005). The human exon 1F and its rat homologue 1_7 have recently received attention in relation to epigenetic regulation in the brain by environmental influences. The promoters 1F/1_7 are sensitive to DNA methylation and this has been linked to a decrease in *Gr* transcript levels both in rodents (Weaver et al., 2004) and in humans (McGowan et al. 2009).

In rodents, the specific post-natal environment such as low maternal care is associated with low levels of adult *Gr* expression in the hippocampus and the paraventricular hypothalamic nucleus (PVN) (Liu et al. 1997). The low *Gr* expression was associated with high methylation of a fully conserved key CpG site (rat CpG 16; human CpG 37) in promoter 1_7 in the hippocampus, corresponding to a binding site of the transcription factor Ngfi-a (also known as Egr-1; zif-268; Krox-24) (Weaver et al. 2004; Weaver et al. 2005; Weaver et al. 2007). Similarly, in humans, up to 40% methylation in the 1F promoter has been observed in suicide victims with a history of childhood abuse (McGowan et al. 2009). However, in peripheral blood mononuclear cells (PBMC's) the key CpG sites (37-38) of the human promoter 1F were methylated only up to 10% or less (Turner et al. 2008), and were highly variable throughout the entire promoter region. In post-mortem brain tissue of patients with major depressive disorder (MDD) or healthy controls, we detected only up to 4% of methylation in the 1F promoter and throughout CpG islands levels were uniformly low (Alt et al. 2010).

In view of the diverse reports on methylation of the *Gr* 1_7 promoter and the lack of direct evidence for the functionality of the Ngfi-a site *in vivo*, we tested *Gr* 1_7 expression and promoter methylation in the context of acute *Ngfi-a* induction in the brain. Adult male Sprague-Dawley rats were subjected to a single immobilisation stress, which is known to

immediately up-regulate *Ngfi-a* levels in the PVN (Watanabe et al. 1994; Umemoto et al. 1997; Girotti et al. 2007). We expected that this would lead to increased *Gr* 1_7 levels.

We found that *Ngfi-a* is immediately up-regulated in the PVN but surprisingly this had no effect on exon 1_7 levels, even in rats with no methylation in the 1_7 promoter. Methylation levels throughout the whole 1_7 promoter were on average below 20%. Comparison of the PVN and hippocampal methylation levels suggests that the hypothalamic methylation was not responsive to a single stressor, but reflected pre-existing inter-individual differences, whilst the more uniform hippocampus was susceptible to acute stress. We suggest that this dichotomy reflects the trait versus state differences in the stress circuitry.

Methods

Animals and acute stress protocol

Ten to twelve weeks old male Sprague Dawley rats were obtained from Harlan (Horst, The Netherlands) and acclimatised for 1 week. Animals were kept under natural light at 22±2 °C and 40±5% relative humidity. Food and water were available *ad libitum*. Animals were randomly divided into 5 groups of 6 animals. Group A was used as an unstressed control group. Animals of group B to E were immobilised for 15 min and decapitated immediately or returned to their home cages for 1-4 hrs before decapitation (Figure 17A). Isolated brains were frozen in isopentane cooled in an ethanol-dry ice bath and stored at -80°C. EDTA anti-coagulated decapitation blood was collected and plasma was stored at -20°C. Plasma corticosterone levels were measured by ^{125}I radioimmunoassay (ImmuChem™, MP Biochemicals; detection limit 5 ng/ml) (Figure 17B). All animal experiments were done in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

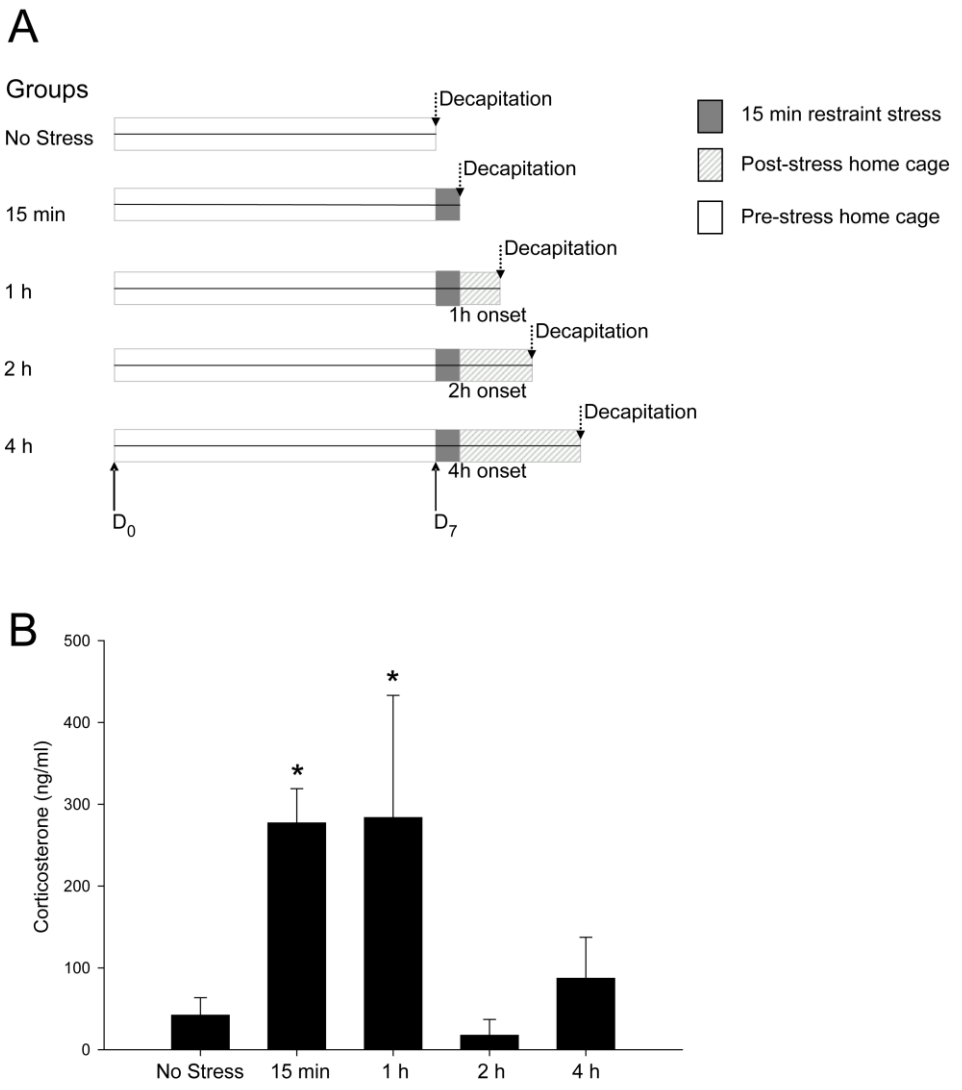

Figure 17:

Restraint stress procedure and plasma corticosterone concentrations after stress.

(A) Rats were either not stressed ("No Stress") or subjected to restraint stress for 15 min. After release they were either immediately decapitated ("15min"), or returned to their home cages before being decapitated 1 to 4 hours after onset of stress. (B) Plasma corticosterone concentrations (mean ± SD) were measured by radioimmunoassay. Significance levels were in comparison to the no stress group. * $P<0.05$.

In situ hybridisation

In situ hybridisation was done using ^{35}S UTP labelled riboprobes as described previously (Meijer et al. 2005). Briefly, from each animal, serial tissue sections were cut in 14µm on a cryostat (Reichert-Jung Frigocut 2800) and thaw-mounted onto Superfrost Plus glass slides and stored at -80 C. The slides were further processed to measure the expression of *Ngfi-a*, *Gr* exon 1_7 and *Gr* total mRNA in the PVN, cortex and hippocampus. The 230 bp *Ngfi-a* probe used was a kind gift of Prof. J. Milbrandt (Washington University, USA). For total *Gr* we used a 500 bp fragment (exon 2, coding for N-terminus of the receptor - courtesy of Dr. M. Bohn, Northwestern University, Chicago, USA) of the original full length rat *Gr* clone (courtesy of Dr. K. Yamamoto, University of California, San Francisco, USA). mRNA containing exon 1_7 was quantified with a specific riboprobe kindly provided by Dr. K. Chapman (University of Edinburgh, UK) (McCormick, Lyons et al., 2000). Sense probes were used as negative controls. After hybridisation, slides were put in a cassette and a Biomax-MR film (Kodak) was exposed for 3 days (*Gr*) to 13 days (exon 1_7) and ^{14}C microscales (GE healthcare, Eindhoven, The Netherlands) were used to calibrate the signal. Films were scanned and quantified using the Image J software (NIH, USA). Average optical densities were corrected for film and tissue background.

Methylation analysis

One section per animal was selected for dissection at room temperature under a stereo-zoom microscope, based on the presence of the PVN and the hippocampus in stained adjacent sections. The hippocampus and a surface of 3x3 mm including bilateral PVNs was scraped into separate RNase-free tubes for subsequent isolation of genomic DNA using the QIAamp® DNA Micro kit (Qiagen, Venlo, The Netherlands) and stored at -20°C. Genomic DNA from hippocampal tissue was isolated using the QIAamp® DNA Mini kit (Qiagen). The EpiTect Bisulphite kit (Qiagen) was used to convert unmethylated cytosine residues to uracil, whereas methylated cytosines remained unmodified. The first two steps of bisulphite treatment were performed on 200-400 ng of genomic DNA. The thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) program comprised several incubation steps for DNA denaturation, sulfonation of unmethylated

cytosine, and deamination of cytosine sulfonate to uracil sulfonate. Cycling conditions were: 1st denaturation for 5 min at 99°C, 1st incubation for 25 min at 60°C, 2nd denaturation for 5 min at 99°C, 2nd incubation for 85 min at 60°C, 3rd denaturation for 5 min at 99°C, 3rd incubation for 175 min at 60°C and hold at 20°C. Alkaline uracil desulfonation and sample purification were performed using a spin column. Modified DNA was eluted and stored at -20°C. The bisulphite-modified DNA was used to amplify promoter 1_7 with the primers and under the conditions shown in Table 6. PCR was performed using 20 mM Tris HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates, 1 x SYBR Green (Cambrex, Verviers, Belgium) and 2.5 U Diamond *Taq* DNA polymerase (Bioline, London, UK) on an Opticon 2 thermal cycler (MJ Research, Landgraaf, The Netherlands). Cycling conditions were as follows: 95°C for 2 minutes, 40 cycles, at 95°C for 20 sec, annealing temperature for 20 sec and 72°C for 25 sec. Products of first round PCRs were used as a template for nested amplifications using the primers and conditions in Table 6. The region homologous to the human *GR* promoter 1A was used as a positive pyrosequencing control. PCR was performed using Platinum *Taq* polymerase (Invitrogen, Paisley, UK) with the primers and conditions described in Table 6. Specific primer sets for rat homologue 1A and all pyrosequencing primers have been selected using the PSQ Assay Design software (Biotage, Uppsala, Sweden). All PCR products were pyrosequenced on a Pyromark ID using Pyrogold reagents (Biotage) to allow quantitative analysis of DNA methylation patterns.

Table 6: Primer sequences and PCR conditions.

methylation analysis	Primer sequences	Tm (°C)	MgCl2 (mM)	Primers (uM)	Product (bp)	DNA Polymerase
rat 1.7 1st round *	fwd: 5'-TTTTTTAGGTTTTTTTAGAGGG-3' rev: 5'-ATTTCTTTAATTTCTCTTCTCC-3'	50	3	0.5	285	D-TP
rat 1.7 nested *	fwd: 5'-TTTTTTTGTTAGTGTGATATATTT-3' rev: Biotin-5'-TTCTCCCAAACTCCCTCC-3'	50	3	0.5	177	D-TP
pyrosequencing primer 1.7	5'-TCTCCCAAACTCCCT-3' 5'-TGATATATTTYGYGTAATTT-3' 5'-GGGGGTTTTGGTTGT-3' 5'-ATTTTAGGGGGTTTTGGTTG-3'					
rat 1A 3'	fwd: Biotin-5'-GGGGTTTTGAAATAATTTGGG-3' rev: 5'-CAAACTACACAACTCTACCCACTT-3'	60	3	0.1	267	Pl-TP
pyrosequencing primer 1A	5'-TTTATGGGTATTTGTTTGAT-3'					

* primers from Weaver et al., Nat Neurosci, 2004
Diamond Taq polymerase: D-TP
Platinum Taq polymerase: Pl-TP

Statistical analysis

All results were analysed using a one way analysis of variance (ANOVA) followed by Tukey post-hoc correction. Data that did not follow a Gaussian distribution were analysed using non-parametric statistical procedures. Data from controls were compared to those of stressed animals using a Kruskal–Wallis test followed by a Dunn's procedure for post-hoc correction. The relationship between promoter methylation and 1_7 expression levels was analysed using Pearson's correlation coefficient. Normal distribution of overall methylation levels in the hippocampus and the PVN was tested using the D'Agostino & Pearson omnibus test. Statistical analyses were performed using SPSS Version 18 (SPSS Inc., Chicago IL, USA) and the Sigma Stat software for Windows (Erkrath, Germany). Differences were considered to be significant when $p<0.05$ after post-hoc correction.

Results

Impact of *Ngfi-a* on *Gr* 1_7 regulation after acute stress

Immediately after 15 min of restraint stress, plasma corticosterone levels measured by radioimmunoassay were up-regulated (Figure 17B). Levels were elevated until 1 hour post stress but returned to pre-stress levels 2 hours after release from the restrainer.

In situ hybridisation revealed that *Ngfi-a* was induced immediately in the rat PVN ($p<0.05$) after 15 min of restraint stress and was still elevated 60 min later (Figure 18A). After 120 min, *Ngfi-a* levels were back to pre-stress levels. Within the 240 min of the experiment the *Gr* transcript 1_7, immediately downstream of the promoter 1_7 containing the Ngfi-a recognition site containing promoter 1_7, was not up-regulated in the PVN (Figure 18B). Total *Gr* was not affected within 4 hours after restraint stress (Figure 18C).

Restraint stress also immediately induced *Ngfi-a* (p=0.002) in the cortex. This induction was short-lived and after 4 hours levels were significantly reduced (p<0.001) below pre-stress levels. As in the PVN, this immediate up-regulation of *Ngfi-a* had no influence on cortical *Gr* 1_7 levels. In the cortex total *Gr* levels remained unchanged.In the hippocampus there was no effect of restraint stress on *Ngfi-a*, exon 1_7, or total *Gr.*

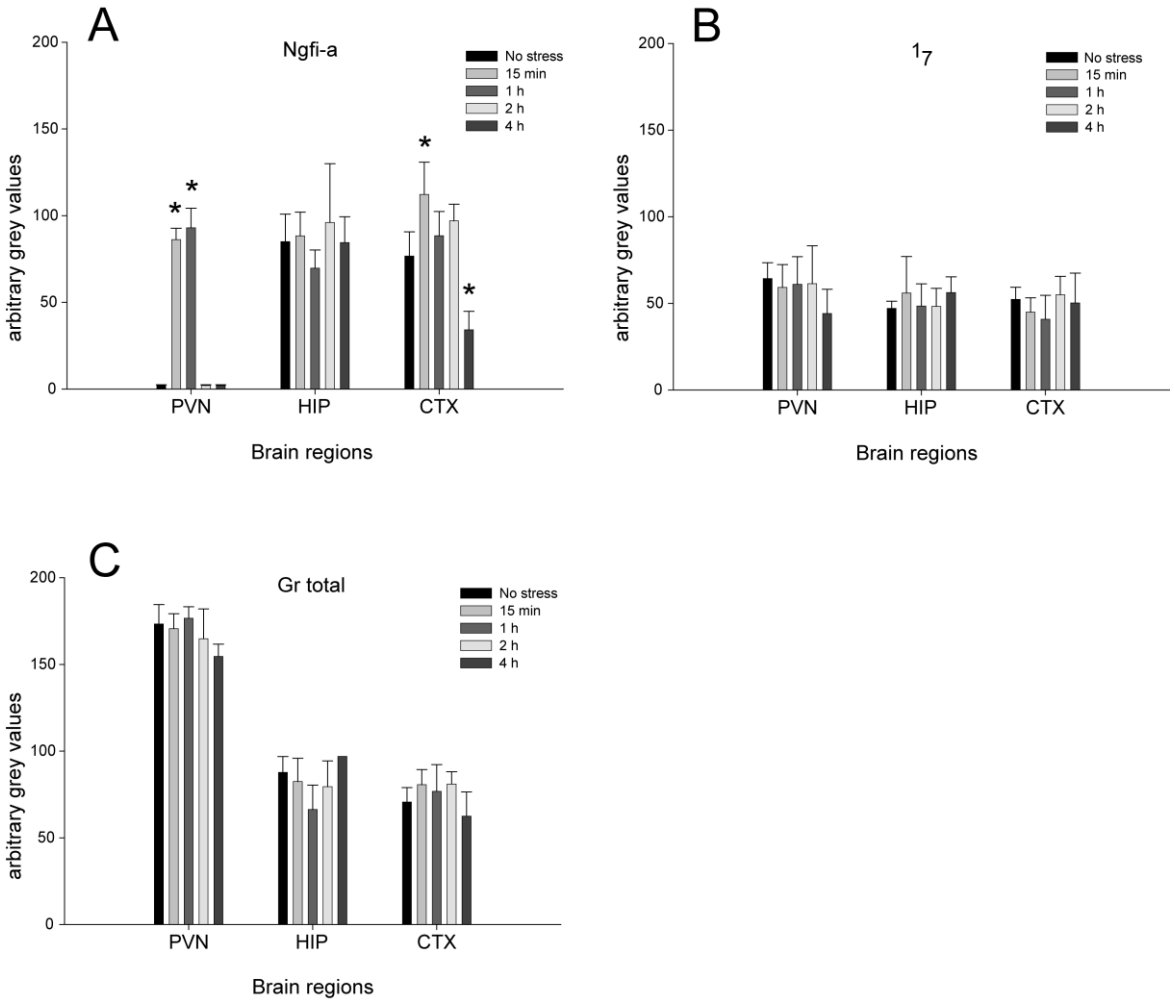

Figure 18:

Ngfi-a does not up-regulate rat 1_7 transcripts *in vivo*.

Quantification of *Ngfi-a* mRNA (A), 1_7 mRNA (B) and total *Gr* mRNA (C) after acute stress by in-situ hybridisation in the paraventricular nucleus of the hypothalamus (PVN), the prefrontal cortex (CTX) and in the CA1 area, representing the hippocampus (HIP). Significance levels were in comparison to the no stress group. * P<0.05.

Methylation of *Gr* promoter 1_7 in the hypothalamus

As described above, *Ngfi-a* levels were immediately induced after acute stress in the PVN (as shown in Figure 18), without up-regulation of 1_7 transcript levels up to four hours later. To test if binding of Ngfi-a to the 1_7 promoter was blocked by DNA methylation, we quantified methylation levels of 15 CpG sites in promoter 1_7. Only CpG 4 showed a significant demethylation ($p = .024$ after post-hoc correction) 60 min and 240 min after acute stress compared to controls (Figure 19) although basic methylation levels were already very low (<5%). Overall methylation levels were below 20% indicating that binding of Ngfi-a to the promoter is not inhibited. However, methylation levels of most CpG sites were highly variable between animals. The D'Agostino & Pearson omnibus normality test revealed that data from the summed methylation is normally distributed in the hippocampus ($K^2 = 12.78$; $p = .0017$) (see below) but not in the PVN ($K^2 = 2.726$; $p = .2559$), reflecting the larger intra- than inter- group differences in the latter region.

The Pearson's correlation coefficients showed no significant link between methylation levels of individual CpG positions and 1_7 transcript levels (r values varied between -.396 and .05 and p values between .068 and .825). However, the sum of methylation levels of all CpG positions showed a significant negative correlation (Figure 20A) with expression levels of the 1_7 transcript ($r = .594$; $p = .004$). This correlation was essentially independent of the first 5 CpG positions (CpG 3-7) suggesting that the methylation of this region does not functionally participate in the regulation of 1_7 transcripts.

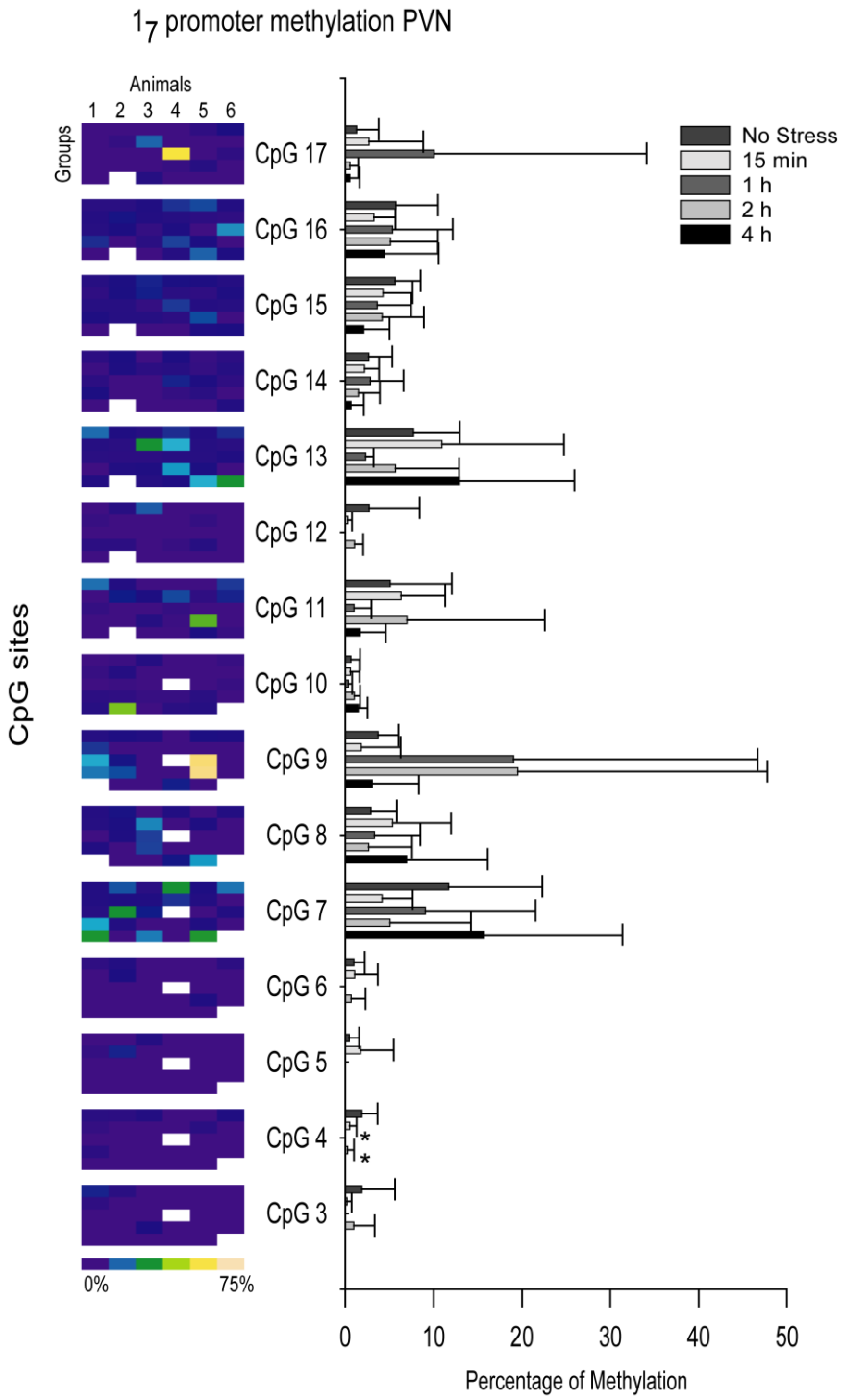

Figure 19:

Methylation analysis of the hypothalamic rat 1_7 promoter.

Percentage of methylation of individual CpG dinucleotides (numbers according to Weaver, 2004) in the 1_7 promoter in the PVN (right panel). Methylation levels are expressed by colour (0% (blue) to 75% (yellow)),

levels from the scale at the left panel bottom. Groups are represented in rows corresponding to the right panel (No Stress-Group in the top row, 4 h-Group in the bottom row). Individual animals are represented in the 6 columns. Significance levels were in comparison to the no stress group. * P<0.05.

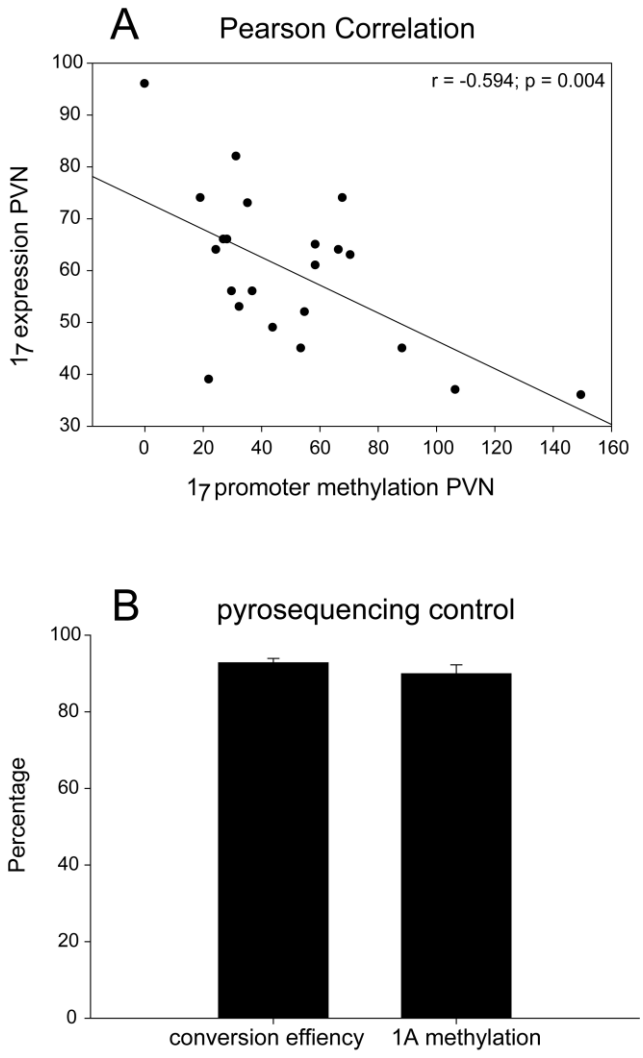

Figure 20:

Correlation analysis in the PVN and high methylation pyrosequencing control

(A) Pearson Correlation between overall 1_7 promoter methylation levels and 1_7 expression levels in the PVN. Pearson correlation coefficient r and p-value are indicated in the panel. (B) The rat homologue of the human 1A promoter which is outside of the CpG island served as a pyrosequencing control to prove that the low methylation levels of the 1_7 promoter are not due to technical pyrosequencing problems. The bisulphite conversion efficiency was 93% and the total average methylation was 90%.

Methylation of *Gr* promoter 1_7 in the hippocampus

Methylation of the hippocampal *G*r promoter 1_7 was also analysed to determine if the 1_7 promoter was available for Ngfi-a binding. In none of the CpG positions of promoter 1_7 methylation levels exceeded 15% suggesting a limited epigenetic influence on Ngfi-a binding to the *Gr* promoter. The Ngfi-a binding site covering CpGs 16-17 was completely unmethylated.

However, 120 min and 240 min after stress, 4 of the 15 investigated CpG dinucleotides showed a significant increase ($p<0.05$) in methylation levels (Figure 21) even after post-hoc corrections. One of these CpGs (CpG 11) suggested by McGowan *et al.* (McGowan et al. 2009) corresponded to a putative NGFI-A binding site in humans (CpG 30-32). The overall pattern of methylation was significantly less variable, but normally distributed ($K^2 = 12.78$; $p = .0017$), in the hippocampus than in the PVN. Methylation levels of neither the individual CpG sites nor the sum of all CpG sites correlated significantly with 1_7 transcripts. No correlation was found between hypothalamic and hippocampal methylation levels.

Pyrosequencing controls

As the methylation levels detected in the hippocampus and the PVN were on average below 20%, we used the region homologous to the human *GR* promoter 1A as a positive methylation control in the pyrosequencing experiments (Figure 20B). This region is outside of the CpG island and therefore highly methylated (89%) (Gruenbaum et al. 1981). The bisulphite conversion efficiency was 93% and the total average methylation was 90%.

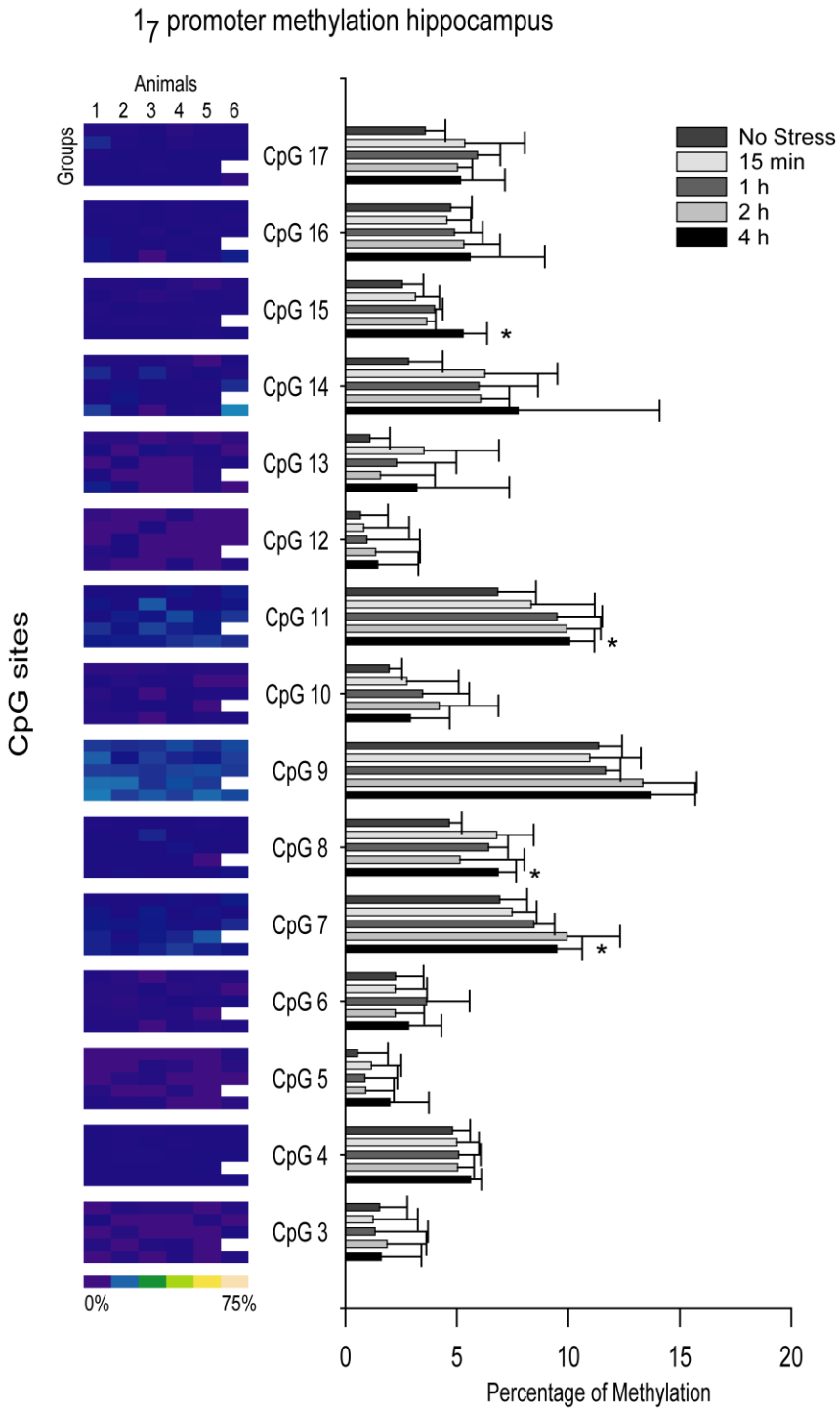

Figure 21:

Methylation analysis of the hippocampal rat 1_7 promoter.

Percentage of methylation of individual CpG dinucleotides (numbers according to Weaver, 2004) in the 1_7 promoter in the hippocampus (right panel). Methylation levels are expressed by colour (0% (blue) to 75%

(yellow)), levels from the scale at the left panel bottom. Groups are represented in rows corresponding to the right panel (No Stress-Group in the top row, 4 h-Group in the bottom row). Individual animals are represented in the 6 columns. Methylation levels for animal 6 of the 2h-Group are missing. Significance levels were in comparison to the no stress group. * P<0.05.

Discussion

When rats were subjected to a 15 min restraint stress, *Ngfi-a* was immediately up-regulated in both the PVN and the cortex but not in the hippocampus. This confirms that *Ngfi-a* is an important immediate early gene at least in these brain regions (Umemoto et al. 1997) and that this transcription factor may play a role in mediating the stress response. Interestingly, this *Ngfi-a* induction did not stimulate 1_7 transcripts in these brain regions, suggesting that it plays either a minor role in 1_7 transcription or that, at least in this context, its activity was blocked. Also, total *Gr* levels were not immediately affected by a single acute stressor. This is in line with previous findings after two hours of restraint stress in both the PVN and the hippocampus (Makino et al. 1995). These and our results are in contrast with the down-regulation of the *Gr* observed after acute stress in the dentate gyrus (DG) (Fujikawa et al. 2000) and the CA1 region of the hippocampus (Yau et al. 2001). Also, after chronic stress, hippocampal *Gr* levels were reliably down-regulated (Makino et al. 1995; Makino et al. 2002) especially in the dentate gyrus (DG) (Yau et al. 2001).

DNA methylation, associated with stable gene silencing (Bird 2007), affects also *Gr* transcription. Early life conditions such as low maternal care lead to high methylation levels in the *Gr* 1_7 promoter in the rat hippocampus (Weaver et al. 2004). The up to 80% methylation levels may have been an overestimation since similar levels have not been observed in other experimental models. In the present study, highly quantitative pyrosequencing (Dejeux et al. 2009) of the 1_7 promoter in the hippocampus after a single acute stress revealed methylation levels not greater than 15%. Our low methylation levels concur with previous reports of hippocampal 1_7 methylation in rats which were either not detectable (Daniels et al. 2009) or below 15% (Herbeck et al. 2010). Similar low levels of methylation were also seen in the human 1F promoter both in newborns

(Oberlander et al. 2008) and adults (Moser et al. 2007; Turner et al. 2008; Alt et al. 2010).

We observed a small but significant increase in hippocampal methylation after a single acute restraint stress in adult rats. This supports the hypothesis that DNA methylation patterns are still variable during adulthood and can be modulated by environmental challenges (Brown et al. 2008). Recently, epigenetic modifications of the *Crf* gene (Elliott et al. 2010) and the *Gndf* gene (Uchida et al. 2011) in the adult mouse brain have been shown to be involved in the regulation of behavioural responses to chronic stress. However, these adaptations are much more subtle than those observed for perinatal programming, and may be collateral to major epigenetic changes elsewhere within the same region or to structural changes e.g. within the chromatin architecture.

Highly variable methylation patterns ranging from 0% to 68% were found in the PVN of different animals. Overall promoter methylation levels, in particular CpGs 7-17 correlated negatively with transcript 1_7 expression, suggesting repressive effects of these methylation events. Since the within–group differences clearly exceeded between–group differences, we interpret these as unrelated to the stressor, but rather as pre-existing individual differences. Thus, environmental influences that were not identified here seem to determine 1_7 promoter activity through methylation of a well defined CpG region. Recently, it has been shown that the amount of maternal care received varies considerably between litter mates and individual care levels correlated positively with hippocampal synaptic plasticity and *Gr* expression levels in this brain region (van Hasselt et al. 2011). Thus, both very early childhood and later changes in the environment during adulthood may lead to epigenetic changes with major effects on brain function. The variation in *Gr* 1_7 methylation as an individual trait is in agreement with the early observations on the effects of maternal care (Liu et al., 1997). However, in the PVN, we found no evidence for a function of the only Ngfi-a binding site identified in the rat promoter 1_7, nor of consequences of its methylation.

In conclusion, although *Ngfi-a* is immediately induced in the PVN after a single acute stress, *Ngfi-a* did not up-regulate *Gr* 1_7 transcript levels as previously suggested (Weaver et al. 2007). We also showed that binding of Ngfi-a to the 1_7 promoter is not blocked by epigenetic modifications such as DNA methylation. Therefore, we suggest that Ngfi-a is not sufficient to drive the expression of natural *Gr* 1_7 transcripts and that

other transcription factors seem to be necessary to control *Gr* 1_7 transcription *in vivo*. Analysis of methylation patterns revealed a striking dichotomy in the PVN and the hippocampus. PVN methylation was not responsive to a single stressor but did exhibit a remarkable individual variation reflecting pre-existing differences. In contrast, in the hippocampus, the epigenetic status is much more uniform but sensitive to acute stress. These results suggest an important differential 'state' versus 'trait' regulation of the *Gr* gene in different brain regions.

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Chapter 6

Conclusions and Perspectives

Conclusions

In this thesis, the differential expression of *GR* and *MR* alternative first exons in multiple areas of the limbic brain system has been demonstrated for the first time in healthy controls and in addition in MDD patients. Hyperactivity of the HPA axis is one of the most consistent findings in MDD as well as impaired HPA feedback which may be due to reduced *GR* or *MR* levels in the forebrain (Pariante 2003; Nemeroff *et al.* 2006). In previous studies it has also been shown that hippocampal *GR* levels can be modulated by epigenetic modifications in *GR* promoters (Weaver *et al.* 2004; McGowan *et al.* 2009).

In the first study, we investigated in post-mortem brain tissues whether the HPA axis hyperactivity observed in MDD may be due to epigenetic modulation of *GR* transcript variants. We hypothesised that in MDD, changes will be observed in alternative *GR* first exon usage and total *GR* expression and that these changes correlate with epigenetic modifications. We showed that distribution patterns throughout all analysed brain regions were similar; nevertheless, we observed significant differences in 5'- and 3'-transcripts between control subjects and MDD patients. *NGFI-A*, a transcription factor of exon 1F was down-regulated in the hippocampus of MDD patients; concomitantly exon 1F expression was reduced. In suicide victims with a history of childhood abuse, 1F transcript levels were reduced and this may be due to methylation of an NGFI-A binding site in this promoter (McGowan *et al.* 2009). However, in MDD, promoter 1F was uniformly unmethylated, indicating that reduced 1F transcript levels are not linked to promoter methylation. Analysing several *GR* promoters for DNA methylation, we confirmed that changes in expression levels were not due to epigenetic methylation of *GR* promoters. We suggested that the susceptibility to depression by inhibition of 1F expression is mediated through promoter methylation in early life adverse events. In MDD promoter 1F usage is also impaired but because of a dearth of *NGFI-A*. These parallel mechanisms towards a similar endpoint may of course be relevant to other genes involved in the pathogenesis of depression.

The MR also plays an important role in HPA axis signalling. The balance between MR and GR levels is thought to be critical for maintaining homeostasis (De Kloet *et al.* 1998). Our second study, conducted together with the Department of Medical Pharmacology at the University of Leiden, The Netherlands, was the first to investigate expression

patterns of two *MR* transcript variants in the human brain and in comparison to MDD patients. The data showed that both *MRα* and *MRβ* mRNA were expressed throughout the human limbic brain with highest expression in the hippocampus, although *MRβ* expression was low. Significantly lower expression levels were detected for total *MR*, *MRα* and *MRβ* in several brain regions of MDD patients compared to controls. This decrease in MDD patients could underlie HPA hyperactivity as well as mood and cognitive disturbances that were often observed in patients suffering from stress-related psychopathologies. Since we found no significant changes in total *GR* expression in MDD but a significant decrease of total *MR* in two brain regions, this would lead to an imbalance of the GR/MR ratio which could further affect HPA axis activity. Limitations of both studies were the small sample size due to limited access to post-mortem brain tissue as well that we cannot exclude that *GR* and *MR* expression may be influenced by antidepressant treatment with tricyclic antidepressants (TCA) and selective serotonin reuptake inhibitors (SSRI). Future studies should include a higher number of subjects of both sexes, preferentially with a known history of stressful life events and similar treatment.

In the first study, we showed decreased levels of exon 1F as well as of *NGFI-A* expression in the hippocampus of MDD patients similarly suggesting a link between *NGFI-A* and *GR* 1F expression also without measurable promoter methylation. This transcription factor was proposed to be involved in the transcriptional regulation of the promoter of the human alternative exon 1F and its rat homologue 1_7 (Weaver et al. 2004; McGowan et al. 2009). Therefore, in the third study, we further investigated the role of NGFI-A in the transcriptional regulation of the *GR in vitro*. Although we confirmed that NGFI-A binds to the 1F promoter and induces the 1F promoter activity in a reporter gene system, we further demonstrated that NGFI-A overexpression does not induce the endogenous 1F transcript. Thus, our data suggest that NGFI-A may be part of the transcriptional complex that activates the reporter gene, but that this is not sufficient to activate the natural 1F promoter *in vitro*.

Gene promoters are rarely dependent on a single transcription factor (reviewed in (Turner et al. 2010)). Analysis of the human *GR* 1F promoter using *in silico* phylogenetic footprinting suggested that NGFI-A is only one of a series of transcription factors that regulates the expression of the 1F transcript (Turner et al. 2008). As NGFI-A was unable to affect endogenous 1F transcription we tested several other transcription factors

predicted to bind the 1F promoter. We found the transcription factors ELK1 and especially E2F1 to strongly up-regulate 1F promoter activity.

The E2F1 binding site covers CpG position 32 that, in human suicide victims with a history of childhood abuse, was highly methylated (40%) in comparison to suicide victims without childhood abuse (15%) (McGowan et al. 2009). Concomitantly, the abused victims had lower 1F transcript levels in agreement with their higher methylation levels. The authors proposed that CpG 32 was part of a second, hypothetical NGFI-A binding site. However, according to our results, we suggest that E2F1 binds in this region and that reduced E2F1 binding to the 1F promoter as a result of DNA methylation could be responsible for the lower 1F expression observed previously. Taken together with our results, we suggest that E2F1 is a major element in the complex necessary to control *GR* 1F transcription *in vitro* and *in vivo*.

Parallel to the third study, we conducted a study in rodents to investigate the role of *Ngfi-a* on *Gr* 1_7 regulation *in vivo*. To naturally induce *Ngfi-a* levels we used the restraint stress paradigm. It has previously been shown that a single restraint stress leads to an immediate increase in corticosterone levels as well as *Ngfi-a* levels (Girotti et al. 2007), an immediate early gene that can be induced within minutes. We demonstrated that 15 minutes of restraint stress significantly induced *Ngfi-a* in the PVN and the cortex, but this had no influence on 1_7 transcript levels. In line with our *in vitro* results (Study 3), we showed that also the rat promoter 1_7 was not transcriptionally regulated by Ngfi-a *in vivo*. Therefore, we suggest that Ngfi-a alone is not sufficient to drive the expression of natural *Gr* 1_7 transcripts and that other transcription factors such as E2F1 seem to be necessary to control *Gr* transcription *in vivo*.

Furthermore, we analysed methylation levels of the 1_7 promoter to exclude that acute stress induced DNA methylation at the Ngfi-a binding site as already shown in association with low maternal care (Weaver et al. 2004; Weaver et al. 2007). We showed that binding of Ngfi-a to the 1_7 promoter is not blocked by epigenetic modifications such as DNA methylation. In the PVN as well as in the hippocampus overall promoter methylation levels were low, <20% and <15%, respectively. However, pyrosequencing of the 1_7 promoter in the PVN revealed a striking variability in methylation of some of the CpG sites (0-68%) and *Gr* 1_7 expression levels correlated negatively with methylation, indicating repressive effects of these methylation events. Recently it has been shown

that the amount of maternal care varies significantly between litter mates and that this correlates with *Gr* expression levels in the hippocampus (van Hasselt et al. 2011). Therefore, we interpret these differences as not related to the acute stress, but rather as pre-existing individual differences. In contrast, methylation levels in the hippocampus were less variable. However, a significant increase in methylation was observed at four CpG sites in the hippocampus four hours after acute stress demonstrating some plasticity of methylation patterns even during adulthood. In conclusion, in the PVN, overall methylation of the promoter showed no changes after stress, but did exhibit a remarkable individual variation, whilst in the hippocampus the epigenetic status showed a more uniform pattern that can respond acutely to changes in the environment such as acute stress. These results suggest a differential 'state' versus 'trait' regulation of the GR gene in different brain regions.

Taken together, the data presented in this thesis show that the regulation of *GR* transcription can be correlated to DNA methylation levels, however, this would appear to be tissue-specific. Although these results suggest a role of DNA methylation in controlling *GR* expression, methylation of single CpG dinucleotides is insufficient to alter *GR* transcript levels or have any physiological consequence.

Perspectives

The results presented in this thesis have gained significant insight in the high complexity of *GR* regulatory mechanisms and the susceptibility of *GR* promoter methylation in response to stress, nevertheless, further in-depth analyses are needed. Future studies should be performed to delineate the transcriptional regulators acting on *GR* transcript expression, including those acting on sequences outside the promoter region. The transcriptional regulation of *GR* 1F probably involves multiple transcription factors and co-activators that have yet to be identified. Also, functional interactions of several factors may be necessary that could be investigated by cotransfection experiments. The use of specific small-interfering RNAs (si-RNA) could help to investigate which transcription factors depending on their combination and the amount of each factor are needed to regulate *GR* 1F transcription.

The alternative *GR* transcripts are not translated into different proteins as the translation start lies within the common exon 2. However, different GR protein isoforms exist, GRα, GRβ and GR-P. It has already been shown that the expression of GRα is preferentially regulated by promoter 1C whilst for the expression of GR-P promoter 1B is predominantly used (Russcher et al. 2007). More research is needed to investigate the link between exons 1D-1J and the resulting protein isoform.

As shown in Chapter 5, the hippocampus seems to be susceptible to 1_7 promoter methylation in response to acute stress, although overall methylation levels were below 15% indicating that the promoter is accessible for transcription factor binding. Further studies should be performed to identify the time frame in which these methylation changes occur since in our study these effects were only followed up until four hours post stress. Methylated DNA immunoprecipitation (MeDIP) could be used to get an overview about the methylation status of the whole rat genome in response to acute and in addition to chronic stress. Using this method, other genes could be identified that might be involved in HPA axis regulation or transcriptional regulators that interact with the GR are also susceptible to methylation. Thus, a more complex overview of epigenetic changes in the genome could be achieved that occur after stress and could add valuable insight in the development of stress-related disorders.

With the work presented in this thesis, another part in the puzzle of the complexity of *GR* transcriptional regulation has been added. Further studies may complete these findings with regard to stress related disorders such as major depression.

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Annexes

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Figure 1: 3

Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis and biological mechanisms that lead to hyperactivity, adapted from (Pariante and Lightman 2008). Circulating cortisol (dashed line) can bind to GRs outside the brain (e.g. pituitary) or inside the brain (e.g. hippocampus and hypothalamus). Activated GRs can induce feedback inhibition to reduce the activity of the HPA axis. Environmental mechanisms such as early life stress and inflammation can affect GR function and in turn impair this feedback inhibition.

Figure 2: 7

Schematic representation of the mineralocorticoid receptor structure.
The N-terminal domain or the A/B region contains the ligand-independant AF-1 site where coactivators can bind to the MR via a non-defined motif. The conserved DNA binding domain (DBD) or C region contains two zinc finger motifs and is responsible for recognition of specific DNA sequences. The variable D region links the DBD to the ligand binding domain (LBD) within the conserved E/F region where coactivators can interact with the MR via a ligand-dependant AF-2 domain.

Figure 3: 9

Schematic representation of MR signaling upon agonist ligand binding.
The MR dissociates from Hsp, which holds it inactive in the cytoplasm, and translocates to the nucleus where it binds to a hormone response element (HRE). Coactivators, such as SRC-1 and p300/CBP, are sequentially recruited to the MR to allow histone acetylation and target gene transcription to occur (Yang and Young 2009).

Figure 4: 11

Structure of the *GR* gene (*NR3C1*; OMIM + 138040; 5q31-q32), the potential mRNA transcripts and the binding sites within the CpG island.
Panel A The genomic structure of the *GR*. ▯ 5' untranslated distal exons; ▨ 5'untranslated CpG island exons; ■ Common exons; ▦ 3' alternatively spliced exons. Panel B shows the potential mRNA transcripts encoding the three GR isoforms: *GRα*, *GRβ* and *GR-P*. Panel C shows the location of the known transcription factor binding sites. ▧ IRF 1 and IRF2 (position 1); ▩ c-Myb, c-Ets ½ and PU1 (position 4); ▩ Ying Yang 1 (positions 5,6,7 and 25); ■ Glucocorticoid response elements (GRE, positions 2, 3, 8, 21, and 22); ■ Sp1 binding sites (positions 9, 10, 12, 13, 16, 19, 20, 21, and 24); □ NGFIA binding site (position 17); ▦ Glucocorticoid response factor-1 (GRF1, position 18); ▤ Ap-1 (position 15); and ▯ Ap-2 (position 23).

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DNA methylation inhibits transcriptional regulation of genes.
A: Cytosine can be methylated at carbon number 5 into 5'-Methylcytosine. B: A complex of transcription factors (TF), the DNA polymerase (Pol) and co-activators (open circles) normally binds to the *GR* promoter and thus regulates gene expression. Methylation of the transcription factor binding site in the *GR* promoter (full ovals) can lead to inhibition of binding to the promoter resulting in reduced expression of the GR. Figure from Alt et al., 2010 with permission.

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Alignment of the rat *GR* promoter 1_7 and the human *GR* promoter 1F.
Solid-lined boxes represent known canonical NGFI-A binding sites either in the rat (Weaver et al., 2004; Daniels et al., 2009; Herbeck et al., 2010) or the human (Moser et al., 2007; Turner et al., 2008; Oberlander et al., 2008; Alt et al., 2010) promoter. The broken-lined box in the human 1F promoter represents a hypothetical non-canonical NGFI-A binding site (McGowan et al., 2009).

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The *GR* and its major isoform variants in selected limbic brain regions.
Expression of the *GR* gene (a). The various 5'-end untranslated exons are spliced to the same splice acceptor site in exon 2. 3'-End alternative splicing results in mRNAs encoding three variants of the *GR* gene: *GRα*, *GRβ* and *GR-P*. Cresyl violet stained sections for histological confirmation of amygdala (Amg), hippocampus (Hi), inferior prefrontal gyrus (IFG), cingulate gyrus (CgG) and nucleus accumbens (Acb) are represented in panel b. Scale bars represent 5 mm. Relative mRNA expression of all *GR* isoforms (c) in different non-depressed brain areas and *GRα* (d), *GRβ* (e) and *GR-P* (f) in control subjects compared to MDD patients. The values are given as $2^{-(\Delta Ct)}$ with the geometric means normalised to *β-actin*. Closed bars represent non-depressed donors; open bars represent patients with MDD. ***** P<0.05.

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GR first exon distribution in the non-depressed and depressed human brain.
Percentage of different *GR* first exons related to the total amount of *GR* in different non-depressed brain areas (a). Percentage of expression levels of *GR* first exons 1B (b), 1C (c), 1D (d), 1E (e), 1F (f), 1H (g) and 1J (h) in control subjects (closed bars) compared to MDD patients (open bars). The values are given as $2^{-(\Delta\Delta Ct)}$ with the geometric means normalised to *β-actin* and related to the total amount of *GR*. ***** P<0.05.

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Transcription factors regulating and internal splicing of, the *GR*.
Relative mRNA expression levels of *GR* regulating transcription factors *YY1* (a), *NGFI-A* (b) and *SP1* (c) in different brain areas. Closed bars represent non-depressed donors; open bars represent patients with MDD. Pearson correlations between mRNA expression levels of *GR* transcript variants, *GR* 3'-isoforms and transcription factors (d-f). Pearson's correlation co-efficient after Bonferroni correction, brain areas and donors are indicated in each panel. ***** P<0.05.

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DNA methylation inhibits transcriptional regulation of genes.
Under normal conditions, a complex containing transcription factors (TF), the DNA polymerase (Pol) and co-activators (open circles) binds to the unmethylated promoter and regulates *GR* expression. If the transcription factor binding site in the promoter is methylated (full ovals), binding is inhibited and thus, *GR* expression is reduced.

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The *GR* promoter region is sparsely methylated in the human brain.
Percentage of methylation was measured by pyrosequencing in 6 patients with MDD and 6 non-depressed donors, covering promoter 1J, promoter 1E, promoter 1B and part of promoter 1F. Methylation levels are expressed by colour (0% (blue) to 100% (yellow)), levels from the scale at the panel top.

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Mean relative number of copies of total *MR* (tMR) and total *GR* (tGR) transcript compared between five brain regions of six 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 normalisation with *tubulin*. Note that expression levels are plotted on a log scale.

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Mean relative number of copies of total *MR* (tMR), *MRα* and *MRβ* within five brain regions of six non-depressed subjects. For each brain region the number of copies of *MRα* and *MRβ* is expressed relative to total *MR* mRNA expression (set at 100%). The

figure presents data after normalisation with *tubulin*. Note that expression levels are plotted on a log scale.

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Mean expression of total *MR* (tMR), *MR*α, *MR*β or total *GR* (tGR) mRNA levels in five 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 *MR*β 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 normalisation with *tubulin*. Note that the expression levels are plotted on a linear scale. * $p < .05$; ** $p < .01$; n.s.= not significant.

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The effect of NGFI-A and E2F1 on human 1F transcript expression.
Relative mRNA expression of *NGFI-A*, exon 1F and total *GR* in 293FT (A) and U373MG (B) cells after transfection with a NGFI-A expression vector was measured by real-time PCR. Relative mRNA expression of *E2F1*, exon 1F and total *GR* in 293FT (C) and U373MG (D) cells after transfection with an E2F1 expression vector was measured by real-time PCR. The values are given as $2^{-(\Delta Ct)}$ with the geometric means normalised to *β-actin*. * $P<0.05$.

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Induction of 1F promoter activity by other predicted transcription factors.

Luciferase activity (mean ± SD) of the 1F reporter gene co-transfected with different transcription factor expression vectors and normalised to the co-transfection with the empty expression vector (pIRES-1F) in 293FT (A) and U373MG cells (B). Western blot analysis of different transcription factors in transfected (T) and untransfected (N) cells to confirm overexpression (C). Chromatin immunoprecipitation (D) of genomic DNA from 293FT as well as U373MG cells using different histone antibodies as markers for inactive (H3K27me3, H3K9me3) or active (H3K4me3, H3K9/14ac) chromatin. Precipitation with a NGFI-A specific antibody (sc-110) was followed by amplification of the 1F promoter region (370bp). Specificity of the antibody was checked by using a blocking peptide (NGFI-A BP) preventing binding of the antibody to the DNA. Chromatin immunoprecipitation with an E2F1 specific antibody (sc-193) was followed by amplification of the 1F promoter region. Normal rabbit IgG and water were used as negative controls whereas the Input was used as a positive control for amplification. * P<0.05.

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Restraint stress procedure and plasma corticosterone concentrations after stress.
(A) Rats were either not stressed (“No Stress”) or subjected to restraint stress for 15 min. After release they were either immediately decapitated (“15min”), or returned to their home cages before being decapitated 1 to 4 hours after onset of stress. (B) Plasma corticosterone concentrations (mean ± SD) were measured by radioimmunoassay. Significance levels were in comparison to the no stress group. * P<0.05.

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Methylation analysis of the hypothalamic rat 1_7 promoter.
Percentage of methylation of individual CpG dinucleotides (numbers according to Weaver, 2004) in the 1_7 promoter in the PVN (right panel). Methylation levels are expressed by colour (0% (blue) to 75% (yellow)), levels from the scale at the left panel bottom. Groups are represented in rows corresponding to the right panel (No Stress-Group in the top row, 4 h-Group in the bottom row). Individual animals are represented in the 6 columns. Significance levels were in comparison to the no stress group. * P<0.05.

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Correlation analysis in the PVN and high methylation pyrosequencing control.
(A) Pearson Correlation between overall 1_7 promoter methylation levels and 1_7 expression levels in the PVN. Pearson correlation coefficient r and p-value are indicated in the panel. (B) The rat homologue of the human 1A promoter which is outside of the CpG island served as a pyrosequencing control to prove that the low methylation levels of the 1_7 promoter are not due to technical pyrosequencing problems. The bisulphite conversion efficiency was 93% and the total average methylation was 90%.

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Methylation analysis of the hippocampal rat 1_7 promoter.
Percentage of methylation of individual CpG dinucleotides (numbers according to Weaver, 2004) in the 1_7 promoter in the hippocampus (right panel). Methylation levels are expressed by colour (0% (blue) to 75% (yellow)), levels from the scale at the left panel bottom. Groups are represented in rows corresponding to the right panel (No Stress-Group in the top row, 4 h-Group in the bottom row). Individual animals are represented in the 6 columns. Methylation levels for animal 6 of the 2h-Group are missing. Significance levels were in comparison to the no stress group. * P<0.05.

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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). 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).

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Primer sequences and PCR conditions.

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Primer sequences and PCR conditions.

Index of Abbreviations

ABN	arched-back nursing
Acb	nucleus accumbens
ACTH	adrenocorticotrophic hormone
AF	activation function
Al	Alzheimer’s disease
Amg	amygdala
ANOVA	Analysis of variance
AP-2	activating protein 2
BP	blocking peptide
BZD	benzodiazepine
CBP	CREB binding protein
CgG	cingulated gyrus
ChIP	chromatin immunoprecipitation
CNS	central nervous system
COPD	compulsory-obsessive personality disturbance
Coup-TF	chicken ovalbumin upstream promoter-transcription factor
CpG	cytosine-phosphate-guanine
CRH/ Crf	corticotropin releasing hormone

CSF	cerebrospinal fluid
Ct	cycle treshold
D	deletion analysis
DBD	DNA binding domain
Dem	dementia
DMEM	Dulbecco's Modified Eagle's Medium
Dep	depression
DG	dentate gyrus
DNA	deoxyribonucleic acid; complementary DNA (cDNA)
dNTPs	deoxyribonucleotides (dATP, dCTP, dGTP and dTTP)
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-fourth edition
EBF1	early B-cell factor 1
EDTA	ethylene diamine tetraacetic acid
E2F1	E2F transcription factor 1
ELK1	ELK1, member of ETS oncogene family
ELL	elongation factor RNA polymerase II
EMEM	Eagle's minimal essential medium
EMSA	electrophoretic mobilityshift assay
F	female

FP	DNAse protection/ DNA footprinting
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
Gndf	glial cell line derived neurotrophic factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRF-1	glucocorticoid receptor DNA binding factor 1
Hal	haloperidol
HC/ HV	healthy control/ healthy volunteer
HES1	hairy and enhancer of split 1
Hi/ HIP	hippocampus
HPA	hypothalamic-pituitary adrenal
HRE	hormone response element
H. sap.	Homo sapiens
11βHSD	hydroxysteroid 11-beta dehydrogenase
HSP90	heat shock protein 90
5-HT	serotonin (5-hydroxytryptamine)
IFG	inferior prefrontal gyrus
Ig	immunoglobulin
IRES	internal ribosome entry site

IRF	interferon regulatory factor
LBD	ligand binding domain
LG	licking-grooming
M	male
Maternal Sep.	maternal separation
MDD	major depressive disorder
MeDIP	methylated DNA immunoprecipitation
Met. Sup.	methyl-supplemented diet
Mo	morphine
MR	mineralocorticoid receptor
NBB	Netherlands Brain Bank
NCoR	nuclear receptor corepressor
ND	no data
NFκB	Nuclear factor kappa-B
NFYC	nuclear transcription factor-Y gamma
NGFI-A	nerve growth factor-induced protein A
P300	E1A binding protein p300
Park	Parkinson disease
Pax3	paired box 3
PBMC	peripheral blood mononuclear cell

PCR	polymerase chain reaction; methylation-specific PCR (MS-PCR)
PFC/ CTX	prefrontal cortex
Pol	DNA polymerase
PPIA	peptidylprolyl isomerase A
PR	protein-restricted
PU.1	spleen focus forming virus (SFFV) proviral integration oncogene spi1
PVN	paraventricular nucleus of the hypothalamus
RFX1	regulatory factor X, 1
RG	reporter gene assay
RHA	RNA helicase A
RIN	RNA integrity number
RNA	ribonucleic acid; messenger RNA (mRNA)
R. nor.	Rattus norvegicus
RT	reverse tanscription
SD	standard deviation
SMRT	silencing mediator of retinoid acid and thyroid hormone receptor
SNP	single nucleotide polymorphism
SP-1	specificity protein 1

SRC	steriod receptor coactivator
SSRI	selective serotonin reuptake inhibitors
TCA	tricyclic antidepressants
TF	transcription factor
UTP	uridine-5'-triphosphate
UTR	untranslated region
WHO	world health organisation
YY1	Ying Yang 1

Index of Publications

This doctoral thesis consists of six chapters. Chapter 1 represents a general introduction and overview including parts that have been published in a 'Review'. Chapters 2-5 were published or submitted for publication as original research in international peer-reviewed journals. Chapter 6 represents a general summary and perspectives. All articles are presented here in the originally published/ submitted form, except for changes in formatting.

Chapter	***has been published as/ submitted for publication as***
Chapter 1 (in part)	Jonathan D. Turner, **Simone R. Alt**, Lei Cao, Sara Vernocchi, Slavena Trifonova, Nadia Battello, Claude P. Muller. Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem Pharmacol. 2010 Dec 15;80(12):1860-68.
Chapter 2	**Simone R. Alt**, Jonathan D. Turner, Melanie D. Klok, Onno C. Meijer, Egbert A.J.F. Lakke, Roel H. de Rijk, Claude P. Muller. Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. Psychoneuroendocrinology. 2010 May;35(4):544-56.
Chapter 3	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. Decreased expression of mineralocorticoid receptor mRNA and its splice variants in postmortem brain regions of patients with major depressive disorder. J Psychiat Res. 2011 Jul;45(7):871-8

Chapter 4 **Simone R. Alt**, Lei Cao, Salomon C. Leija, Sophie B. Mériaux, Onno C. Meijer, Jonathan D. Turner, Claude P. Muller. Transcriptional regulation of the glucocorticoid receptor transcript 1F: NGFI-A and E2F1? Submitted.

Chapter 5 **Simone R. Alt**, Jonathan D. Turner, Ans M.I. Tijssen, Onno C. Meijer, Claude P. Muller. Dichotomy in methylation patterns and sensitivity to acute stress of glucocorticoid receptor promoter 1_7 in the hypothalamus and the hippocampus. Submitted.

Erklärung

nach § 9, Abs. 1 der Promotionsordnung des Fachbereichs I der Universität Trier vom 13.11.2008.

Hiermit versichere ich, dass ich die vorliegende Dissertationsschrift selbständig verfasst und keine anderen als die angegebenen Hilfsquellen verwendet habe. Diese Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Luxemburg, im März 2010

Dipl. Biol. Simone Regina Alt