Stimulus-dependent glucocorticoid receptor signaling in early-life stressed mice

Dissertation der
Fakultät für Biologie der Ludwig-Maximilians-Universität München

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München
2014

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Tag der Einreichung: 11. December 2014

Tag der mündlichen Prüfung: 18. Mai 2015

Dla mojej córeczki Kaji, męża Michała

i całej rodziny.

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List of Abbreviations

5'UTR	5' untranslated region
5hmC	5' hydroxymethyl cytosine
5hmU	5' hydroxymethyl uracil
5mC	5' methyl cytosine
5caC	5' carboxylcytosine
5fC	5' formylcytosine
ACTH	adrenocorticotropic hormone
AID	activation-induced (cytidine) deaminase
AP-1	activator protein 1
ABN-LG	arch-back nursing and licking/grooming
APOBEC	apolipoprotein B editing complex
ATP	adenosine-5'-triphosphate
Atp5j	ATP synthase, H+ transporting, mitochondrial F0 complex
AVP	arginine vasopressin
BER	base excision repair
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
C	cytosine
CGI	CpG island
ChIP	chromatin immunoprecipitation
CpG	cytosine guanine dinucleotide
CRH	corticotropin-releasing hormone
Ctrl	control
DBD	DNA binding domain

ddH_2O	di-distilled water
DEPC	diethylpyrocarbonate
Dex	dexamethasone
DG	dentate gyrus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
dNTP	deoxyribonucleotide
Drr1	down-regulated in renal cell carcinoma 1
Dusp1	dual specificity phosphatase 1
ELS	early-life stress
Fkbp5	FK506 binding protein 51
fwd:	forward
G	guanine
G6pdx	glucose-6-phosphate dehydrogenase X-linked
Gilz	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
GRE	glucocorticoid response element
hGR	human glucocorticoid receptor
HR	hinge region
HSP	heat shock proteins
IHC	immunohistochemistry
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilo base
LBD	ligand binding domain
MDD	Major depressive disorder

MR	mineralocorticoid receptor
mRNA	messenger RNA
MS	maternal separation
MeCP2	methyl-CpG-binding protein 2
ND	not determined
NGFI-A	nerve growth factor-induced protein A
NL	nuclear localization
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdk4	pyruvate dehydrogenase kinase, isozyme 4
PFC	prefrontal cortex
PND	postnatal day
POMC	proopiomelanocortin
PVN	paraventricular nucleus
qPCR	quantitative PCR
rev:	reverse
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcription PCR
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
Sgk1	serum glucocorticoid kinase 1
s/m/h	second /minute/ hour
Sp1	specificity protein 1
SSC	saline-sodium citrate
T	thymidine

TBS	tris-buffered saline
TE	tris-EDTA buffer
TET	ten-eleven-translocation
X-gal	bromo-chloro-indolyl-galactopyranoside
YY1	Yin Yang 1

Abstract

Epigenetic programming facilitates the adaptation of an organism to changes in the environment through lasting alterations in gene expression that underlie certain physical and behavioral phenotypes. Exposure to adverse events in early postnatal life is known to increase the risk for stress-related psychiatric disorders later on. Our previous studies showed that early-life stress (ELS) in mice caused by periodic infant-mother separation (MS) leads to increased hyperactivity of the HPA axis, reduced glucocorticoid feedback inhibition, and depressive-like behavior. Moreover, our work revealed ELS-induced hypomethylation of the arginine vasopressin (*Avp*) gene enhancer and pro-opiomelanocortin (*Pomc*) promoter.

The aim of the study was to investigate whether ELS can also lead to epigenetic programming of the mouse glucocorticoid receptor (GR*, Nr3c1*). GR is a major feedback regulator of the hypothalamic-pituitary adrenal (HPA) stress axis and its expression is regulated by multiple promoters associated with its5' untranslated first exons. Given the fact that the mouse GR promoter was only partly characterized, we aimed to determine its genomic structure. In addition, tissue distribution and absolute quantification of newly identified alternative first exon transcripts were analysed. Although most of the first exon transcripts were found to be widely expressed, some of them are shown to be differentially regulated by growth factor- and depolarization-induced signaling.

In the present work we show also that mice with a history of maternal separation display up-regulated *GR* mRNA levels. This observation was confined to Crh-producing neurons in the hypothalamic paraventricular nucleus (PVN), which are principal effectors of the stress response. Moreover, elevated levels of GR are shown to be responsible for stronger induction of its downstream target genes (*Fkbp5*, *Sgk1,* and *DUSP1*), which suggests an enhanced transcriptional activity of the GR in ELS mice. This effect is supported by a higher occupancy

of the GR at the glucocorticoid response elements (GREs), following corticosterone injection (i.p.).

Finally, we report here that an enhanced level of GR expression in ELS mice is accompanied by an increased methylation of specific CpG residues at the CpG island shore region of the *GR* promoter. These ELS-responsive CpGs comprise a DNA binding site for the transcriptional repressor Yin Yang 1 (YY1). Given the high homology of the mouse and human *GR* promoter, and the conservation of the YY1 binding site, we conducted a methylation analysis of the hGR CpG island shore region in peripheral tissues and post mortem brain samples. Our findings might serve as a basis for comparing the methylation patterns in tissues from control subjects and patients with stress-related brain disorders.

CHAPTER 1 INTRODUCTION

1.1 The role of epigenetic mechanisms in psychiatry

Epigenetics refers to the study of changes in gene expression that occur independently of alterations in primary DNA sequence. Importantly, epigenetic modifications remain stable during cell division and can be inherited across generations (Bird *et al.* 2007). Changes at the epigenome are essential for all eukaryotic organisms and are key mechanisms to control biological processes such as:

- ✓ genome reprogramming during early development (Li *et al.* 2002; Reik *et al.* 2007);
- ✓ cellular differentiation in order to establish cell- and tissue-specific gene expression patterns. This leads to phenotypically different tissues, although each cell in the body harbors the same genetic information (Ng *et al.* 2008);
- ✓ adaptation of the developing and mature organism to a wide range of environmental cues (e.g. stress, nutrition or drugs) (McGowan *et al.* 2009; Murgatroyd *et al.* 2009; Weaver *et al.* 2004);
- ✓ aging, which is associated with accumulation of epigenetic alterations over lifetime and contribute to a progressive decline in the function of adult tissues (López-Otín *et al.* 2013).

Major epigenetic mechanisms involved in gene regulation are:

- ✓ direct postsynthetic chemical modification of DNA by cytosine methylation (and demethylation) in the context of cytosine-guanine dinucleotides (CpGs) (described below) (Jaenisch *et al.* 2003);
- ✓ post-translational modifications of histone proteins such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation, which modulate chromatin structure and are associated with transcriptional states (open, transcriptionally

active chromatin, so called euchromatin, versus closed, inactive chromatin, so called heterochromatin) (Portela *et al.* 2010);

- ✓ small non-coding RNAs (microRNAs), which posttranscriptionally control the expression of their target genes by mRNA degradation or blocking of translation (Chuang *et al.* 2007; Sato *et al.* 2011)

Together, these processes orchestrate transcriptional activation or repression of genes in a context-dependent manner through changes in chromatin structure. Hereby, they determine the DNA accessibility to various proteins including those that recognize more directly such modifications and upon DNA binding and/or association with the chromatin confer transcriptional regulation (Jaenisch *et al.* 2003; Costa *et al.* 2008). While epigenetic modifications are required for normal development and an organism's health, they can also contribute to pathological states. Defects in the epigenetic machinery have been linked to various disorders, including cancer, psychiatric, neurological and autoimmune diseases (Bredy *et al.* 2010; Kaiser *et al.* 2010; Portela *et al.* 2010; Murgatroyd *et al.* 2012)

1.1.1 DNA methylation and demethylation

In mammalian cells, DNA methylation is typically found at cytosine residues located at the 5' position of the pyrimidine ring, in the dinucleotide 5′-CpG-3′ (Suzuki *et al.* 2008). Addition of a methyl group from S-adenosyl methionine (SAM) to cytosine is catalyzed by a family of DNA methyltransferases (Dnmts). Three members of this family contain enzymatic activity: Dnmt1, Dnmt3a, and Dnmt3b (Li *et al.* 2002). Ubiquitously expressed Dnmt1 is a maintenance methyltransferase that shows preference for hemi-methylated DNA, and initiates methylation of the complementary strand after DNA replication. Its role is to maintain pre-existing methylation patterns in proliferating cells (Bird *et al.* 2002). *De novo* methylation is

mediated by Dnmt3a and Dnmt3b, which add methyl groups to previously unmodified DNA. These enzymes are highly expressed in early embryonic cells, down regulated after differentiation, and expressed at low levels in adult differentiated somatic cells. Dnmt3a and Dnmt3b are essential for normal development (Okano *et al.* 1999; Wu *et al.* 2010).

Although, DNA methylation is a comparatively stable epigenetic mark when compared to histone modifications, accumulating evidence indicates its highly dynamic nature. The appropriate balance between methylated and unmethylated CpG residues is crucial for an organism's health. At some developmental stages, e.g.; in the zygotic paternal genome during embryonic development, rapid DNA demethylation has been observed (Wu *et al.* 2010). Although enzymes which carry out DNA methylation are well defined, the pathway leading to the removal of a methyl group is not yet completely understood. It has been suggested that DNA methylation can be reversed by two distinct pathways, namely passive or active demethylation (Figure 1). Passive demethylation occurs by lack of *de novo* methylation following DNA replication and consequently leads to a dilution of this modification (Chen *et al.* 2011*)*. On the other hand, active demethylation was proposed to be independent of cell division (Guo *et al.* 2011). Direct removal of the 5-methyl group from 5-methylocytosine (5-mC) seems to be very unlikely, due to the high energetic barrier (Kohli & Zhang 2013), however, several potential multistep enzymatic pathways have been proposed. Recent reports suggest hydroxylation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), a reaction catalyzed by the family of ten-eleven translocation (TET) proteins, to represent a potential pathway. Subsequently, deamination is catalyzed by AID/APOBEC to convert 5-hmC to 5-hydroxymethyl uracil (5hmU) for further processing by thymine DNA glycosylase (TDG) and the base excision repair machinery (BER) to regenerate unmodified cytosine (Wu *et al.* 2010). It has also been reported that TET-mediated enzymatic oxidation of 5mC to 5hmC may lead to the formation of 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC),

which then can be removed by the DNA repair pathway (Kohli *et al.* 2013). Moreover, it has been suggested that 5hmC contributes to passive demethylation, because is not recognized by DNMT1 and methyl-CpG binding proteins (Valinluck *et al.* 2007).

In addition to a role in proliferating cells, the importance of dynamic DNA methylation changes has also been expanded to post-mitotic tissues, such as the brain. Post-mitotic neurons express DNMTs, which mostly decline after differentiation in other tissues (Moore *et al.* 2013). Importantly, active *de novo* methylation and demethylation may occur in mature neurons in response to various cues, including behavioural stimulation (Guo *et al.* 2011).

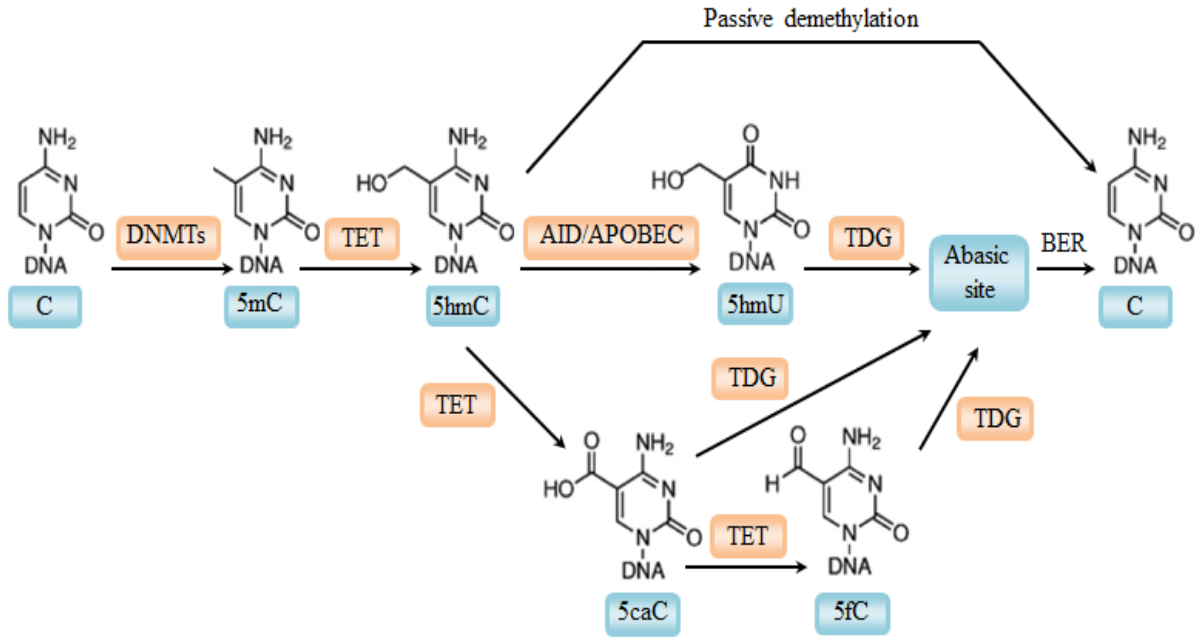

Figure 1: Potential mechanisms for active DNA demethylation
DNA methylation is established and maintained by DNA methyltransferases (maintenance DNMT1, and de novo DNMT3a and DNMT3b). DNA demethylation may occur via passive (e.g.; during replication) or active mechanisms. Active DNA demethylation is thought to involve hydroxylation of 5-metylocytosine (5mC) to 5-hydroxymetylocytosine (5hmC) by ten-eleven translocation (TET) proteins. 5hmC can be next deaminated to 5-hydroxymethyluracil (5hmU) by Aid/APOBEC deaminases. Alternatively, 5mhC can be converted by TET to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). In the end 5mhU, 5fC and 5caC are removed by glycosylases, such as TDG, and replaced with unmodified cytosine. 5hmC contributes to replication independent passive DNA demethylation, due to poor recognition by DNMT1. Adopted from (Wu *et al.* 2010).

Almost 80 % of all CpG sites are methylated and their genomic location often determines their methylation status. Across the mammalian genome, CpG dinucleotides are not randomly distributed, but often cluster in regions known as CpG islands (CGIs). CGIs are defined as regions of DNA with an average length of 200 – 4,000 bp, an CG content > 50%, and a ratio of observed CpG to expected CpG ≥65%. Typically, CpG islands are unmethylated, and are present within or close to the proximal promoter region of genes and often contain transcriptional starts sites (TSS). Approximately 60% of the mouse and human gene promoters are associated with an CGI (Bird *et al.* 2002; Deaton *et al.* 2011). For the majority of the genes, CpG island methylation leads to transcriptional repression. However, CGI methylation does not always reflect the level of expression (Bird *et al.* 2002). For example, genes that are tissue-specifically expressed may remain unmethylated in tissues, where the gene is not expressed (e.g.; MyoD in non-muscle tissues) (Bustos *et al.* 2009). In some cases, DNA methylation can lead to transcriptional activation of genes (e.g.; methylation at the imprinting control region of the insulin-like growth factor 2 gene at the paternal allele can activate its transcription) (Bartolomei *et al.* 2011).

There are two primary mechanisms by which DNA methylation affects gene expression. Methylated cytosine residues can serve as a binding site for a variety of methyl-CpG-binding proteins (MBDs), which recruit additional proteins, e.g.; histone deacetylases (HDACs) or histone methyltransferases (HMTs). These proteins harbor enzymatic activities, which promote the formation of a condensed chromatin structure and lead to the suppression of gene expression. Alternatively, a methyl group can interfere with the recognition of DNA sequences by specific transcriptional factors at their binding site within gene regulatory regions (Bird *et al.* 2002; Li *et al.* 2002).

Although, most studies focused so far on CGI methylation, recent findings revealed an additional relationship between gene expression and DNA methylation at a region termed

"CpG island shore". CpG island shores are defined as regions of lower CpG density, which are located nearby CGIs (up to 2 kb upstream) and methylation at these site seems to occur in tissue-specific manner (Doi *et al.* 2009; Irizarry *et al.* 2009).

1.1.2 Gene-environment (G x E) interactions

It is well known that an individual's phenotype does not only depend on its genetic background, but also on the synergistic interactions between genes and environmental cues. Recent studies indicate that epigenetic modifications can fulfill a role as mediator, which facilitates the adaptation of the organism to signals from the environment (including stress, smoking, physical activity, or diet, among others) at different periods in life (Figure 2). In contrast to the genome, which is rather stable over lifetime, the epigenome can be dynamically modulated by environmental factors, a phenomenon known as "environmental programming" (Murgatroyd *et al.* 2009). Hence, accumulation of epigenetic marks throughout life results in enduring alterations in expression of different genes and in consequence can modulate an adult physical and behavioural phenotype (López-Otín *et al.* 2013).

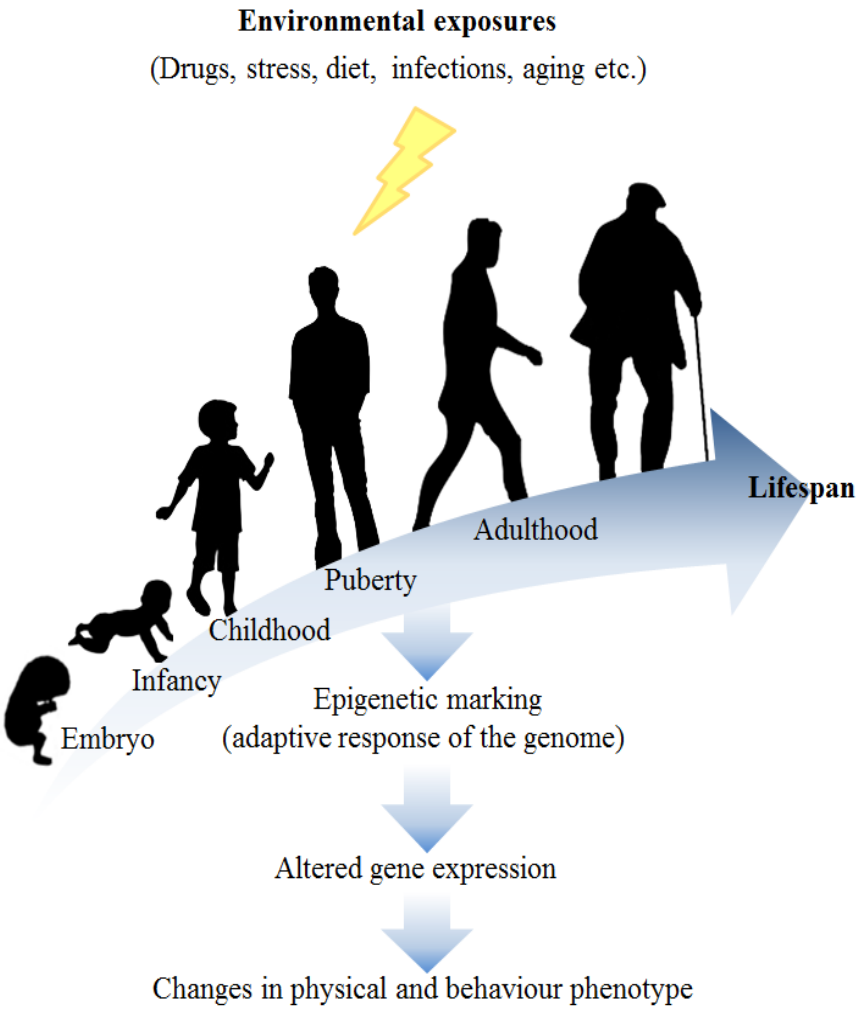

Figure 2. Gene-environment interactions
Prenatal, postnatal, pubertal, and/or adult exposure to environmental factors have the potential to induce changes in the epigenome. This can lead to stable alterations in gene expression and consequently affect the phenotype later in life.

This view is supported by the studies of monozygotic twins (MZ), which have identical genomes, but distinct phenotypes. Epigenetic differences between MZ twins develop after birth and increase with age (Fraga *et al.* 2005). Some phenotypic variations of MZ twins, including the manifestation of psychiatric diseases in one of two genetically predisposed twins, may be potentially influenced by gene-environment interactions (Fraga *et al.* 2005; Petronis *et al.* 2006; Czyz *et al.* 2012). In this respect, valuable information is provided by studies of animals with identical genotypes. For instance, a well- known example of gene-environment interaction is the agouti mouse model. In these mice, the color of the fur is

associated with the degree of methylation at the intracisternal A particle element (IAPs) at the promoter region of the *agouti* gene (the unmethylated state leads to yellow coat, whereas the methylated state leads to brown coat color). Interestingly, the methylation status of the IAP can be modulated by maternal nutrition. For example, a diet rich or poor in methyl donors, affects regulation of *agouti* expression in the offspring, as indicated by the color of the offspring's coat (Dolinoy *et al.* 2008).

1.1.3 Early-life stress (ELS)

Gene-environment interactions during the pre- and postnatal period are especially important in shaping later adult phenotype. Studies in animals and humans have shown that different early life events, in particular stress, may affect the developing brain and promote long lasting changes in neural structure and function. ELS (e.g.; sexual or psychical abuse, lack of parental care) is an important risk factor for the development of stress-related psychiatric disorders, such as anxiety, major depression (MDD), and post-traumatic stress disorders (PTSD) (Cutajar *et al.* 2010; Heim *et al.* 2012; Labonte *et al.* 2012; Lupien *et al.* 2009; Raabe *et al.* 2013). Child abuse has been shown to be correlated with more frequently occurring suicide attempts in adulthood (McGowan *et al.* 2009), cognitive difficulties (Lupien *et al.* 2009), alcohol and substance abuse (Anda *et al.* 2006) or inappropriate aggression (Ford *et al.* 2010). Altogether, these findings suggest that the postnatal period is an especially vulnerable time window during which the brain is highly plastic and sensitive to long-term programming (Lupien *et al.* 2009). However, the molecular mechanisms mediating these effects are still poorly understood.

In order to investigate human traumatic events and to better understand the consequences of early life adversity, different animal models have been proposed. Given the importance of postnatal maternal care in rodents, a common approach to induce ELS is the manipulation of

the relationship between the pups and their mother. Periodic infant-mother separation (MS) is a well-established paradigm which is applied for 3 hours per day, starting at post-natal day 1 (PND1) and terminated about PND10 in mice (PND2- PND14 in rats) (Lupien *et al.* 2009; Murgatroyd *et al.* 2009). Maternal separation is reported to be associated with long lasting hyperactivity of the HPA axis, impaired learning and memory, and an increased depression-like behaviour in adulthood (Murgatroyd *et al.* 2009). Another approach to test the effects of ELS is maternal deprivation (MD), a single 24-h episode applied during the first 2 weeks of life. This paradigm is shown to induce hyperactivity of the HPA axis (Chen *et al.* 2012), affect the rat's hippocampal structure (Oomen *et al.* 2010), and to modify cognitive functions in adulthood (Oitzl *et al.* 2000). Lastly, as shown by Meaney and colleges, Long-Evans rat dams can be classified either as high- or low licking and grooming (LG) and arched-back nursing (ABN) mothers due to naturally occurring variations in maternal care. In this model, the quality of maternal care has a strong impact on offspring's anxiety behaviour in adulthood (Caldji *et al.* 1998) and epigenetic status of the glucocorticoid receptor gene promoter (Weaver *et al.* 2004) (for details see section 1.3.4 " Epigenetic programming of GR").

Furthermore, it is important to note that some effects of adverse early life events are reversible through appropriate, timely interventions (e.g.; pharmacological treatment or behavioural manipulations). For example, foster care applied in mid childhood, was shown to improve partly cognitive defects of young institutionalized children (Smyke *et al.* 2010). Furthermore, the cross-fostering method used in Long-Evans rats (offspring of low-LG-ABN mothers reared by high-LG-ABN dams) was able to reverse the endocrine and behavioural responses to deficits in maternal care (Weaver *et al.* 2004). These observations support the important influence of maternal parenting behavior on the offspring's epigenome.

Moreover, the study of Murgatroyd *et al.* (2009) revealed that chronic application of the selective AVP V1b (SSR149415) receptor antagonist partly normalized ELS-induced neuroendocrine and behavioural phenotypes in adult mice (Murgatroyd *et al.* 2009). Lastly, supplementation with the amino acid L-methionine (a precursor of SAM) reversed the effect of variations in maternal care on epigenetic marking of *GR* and HPA-axis responsiveness to stress in rats (Weaver *et al.* 2005).

1.2 The hypothalamic-pituitary-adrenal (HPA) axis coordinates the response to stress

Stress induces multiple biological responses (e.g.; neuronal, hormonal and immunological) which allow an organism to cope with and adapt to a challenge. An important part of this response is the activation of hypothalamic–pituitary–adrenal (HPA) axis (Figure 3). Briefly, following a physical or emotional stress neurons in the parvocellular division of the hypothalamic paraventricular nucleus (PVN) secrete two types of peptides into the hypothalamo-hypophysial portal system: corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These factors stimulate the anterior pituitary to synthesize pro-opiomelancortin (POMC), which is the precursor of adrenocorticotropic hormone (ACTH), among other peptides. AVP on its own has little potential to stimulate ACTH secretion, whereas the synergistic interaction with CRH regulates efficiently ACTH release (Charmandari *et al.* 2005). ACTH, secreted into the bloodstream, induces release of corticosteroids (cortisol in human, corticosterone in rodents) from the adrenal glands (Aguilera, 2011). Glucocorticoids (GCs) are small steroid molecules which are able to easily penetrate through cell membranes into every cell of the body, and subsequently exert their effects by binding to two specific, ligand-gated receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Both receptors reside in their unbound state in the cytosol and translocate upon binding of their respective ligands to the nucleus where they act

as transcription factors. Hereby, the MR shows an about 10-fold higher affinity to GCs than the GR (de Kloet *et al.* 2005). Thus, the MR is predominantly occupied at low GC levels, while the GR needs higher GC concentrations to be fully activated (Zanchi *et al.* 2010). Moreover, both receptors display different expression patterns: GRs are widely expressed with high levels in pituitary corticotropes and throughout the brain, especially in the hippocampus and hypothalamic neurons, while MRs are mainly limited to the hippocampus and to lesser extend to the hypothalamus (Groeneweg *et al.* 2011; Goncharova *et al.* 2013).

Following activation, the stress system is inhibited at multiple sites in the brain (hippocampus, cortex, and hypothalamus) and the anterior pituitary. GCs as an end product self-limit their secretion through corticosterone-mediated negative feedback mechanisms (Figure 3). Prolonged activation of the HPA axis and sustained secretion of cortisol (corticosterone) become harmful for peripheral tissues and the brain. Although, glucocorticoids are absolutely necessary for many vital functions and prepare the organism for proper endocrine, metabolic and behavioural responses to environmental challenges, their high levels across long time periods may result in pathological states and subsequently lead to health problems later in life, including obesity, insulin resistance, osteoporosis, cardiovascular problems, as well as mood and cognitive impairments (de Kloet *et al.* 2005). Thus, efficient activation of the stress response as well as its termination are necessary to prevent the development of pathological states.

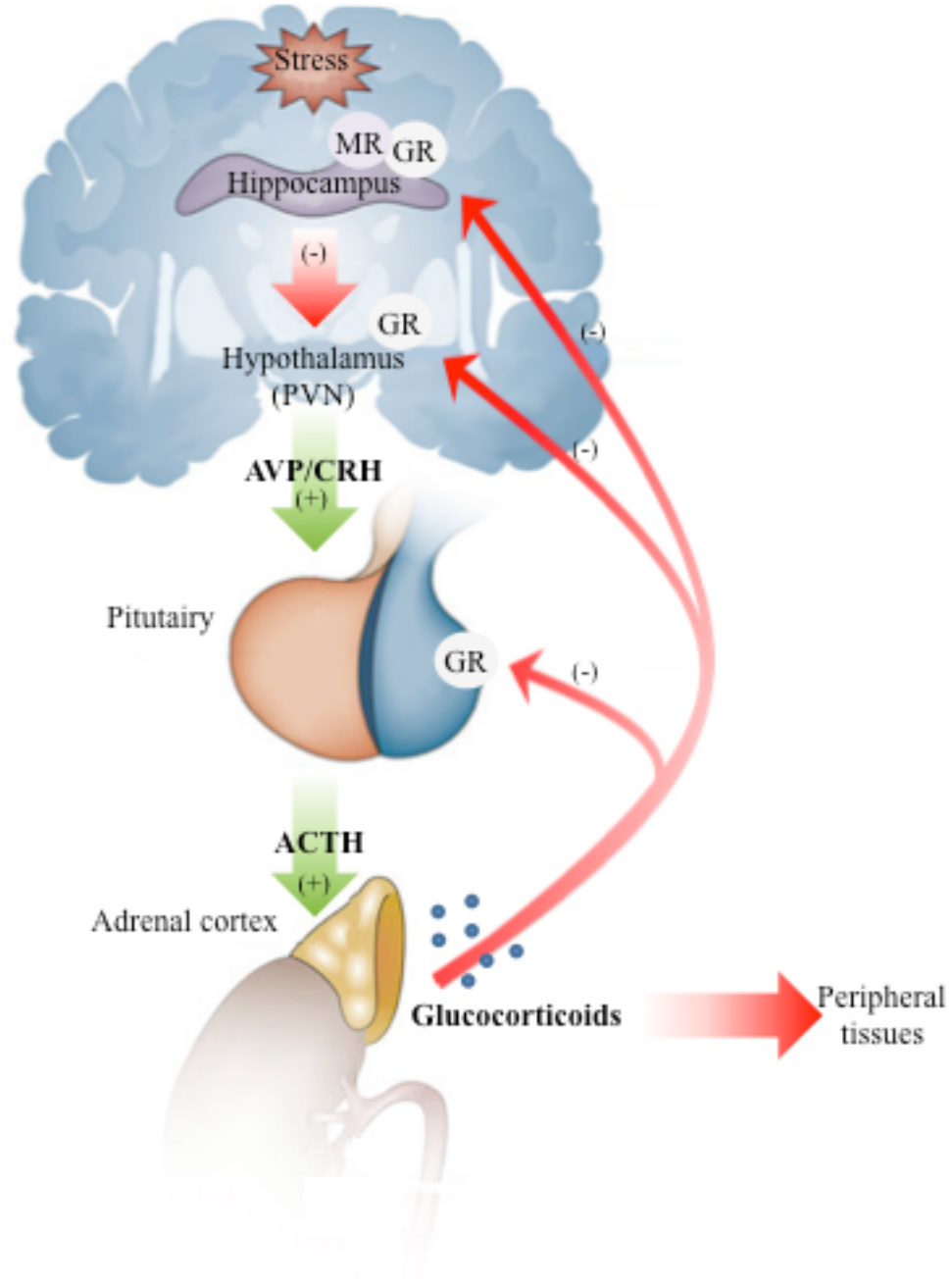

Figure 3: Regulation of HPA axis in response to stress
Activation of the HPA axis leads to secretion of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamic PVN. These neuropeptides stimulate the anterior pituitary to release adrenocorticotropin (ACTH), which in turn acts on the adrenal cortex to secrete glucocorticoids (cortisol in human or corticosterone in rodents). Glucocorticoids (GCs) exert their effect by binding to glucocorticoid (GR) and mineralocorticoid receptors (MR). GCs act back on the hippocampus, hypothalamus, and pituitary gland to attenuate the stress response by a negative feedback loop. The positive (+) forward regulation of the HPA axis is shown in green; while the negative (-) feedback regulation by GRs is indicated in red. Adopted from (Papadopoulos *et al*. 2012)

Clinical studies reported that hyperactivity of the HPA axis, characterized by basal hypercortisolemia, is detected in a large proportion of patients suffering from mood disorders (e.g.; MDD) (Swaab *et al.* 2005). These conditions are thought to be mediated (at least in part) by an impaired negative feedback inhibition, possibly due to an altered function or expression of GRs in the hippocampus and/or the hypothalamus (Holsboer *et al.* 2000; Pariante *et al*. 2001; Zunszain *et al.* 2011). Indeed, post mortem studies revealed reduced levels of *GR* mRNA in the hippocampus of suicide completers with a history of childhood abuse (in comparison to suicide victims with no history of childhood abuse and control subjects) (Labonte *et al.* 2012). The glucocorticoid resistance hypothesis is also supported by results of the dexamethasone suppression (DST) or dex/CRH test (Holsboer *et al.* 2000). Dexamethasone (dex) is a potent synthetic glucocorticoid, which specifically binds to GRs, poorly penetrates the blood brain barrier (BBB) in mouse and human, and thus acts primarily at the pituitary level to inhibit ACTH and corticosteroids secretion (de Kloet *et al.* 2005). For example, Heim *et al.* (2008) have reported that dex administration leads to increased secretion of ACTH and cortisol in MDD patients with a history of childhood abuse [in comparison to non-abused/MDD and control subjects (Heim *et al.* 2008)]. All these reports suggest that early traumatic events affect the developing HPA axis and increase the susceptibility to psychiatric disorders later in life.

This view is supported by a number of rodent studies, although results obtained from animals are difficult to be extrapolated to humans. Murgatroyd *et al.* (2009) have shown that ELS in male mice leads to hyperactivity of the HPA axis, both under resting conditions and in response to an acute stressor, as indicated by increased plasma corticosterone levels. In addition, dexamethasone did not suppress plasma corticosterone levels in 3 month old mice with a history of ELS as compared with controls. Moreover, ELS mice display enhanced levels of *Avp* in the PVN (Murgatroyd *et al.* 2009) and pituitary pro-opiomelancortin (*Pomc*)

(Y. Wu *et al.* 2014) throughout life. Changes in *Avp* expression associate with CpG hypomethylation at the *Avp* enhancer, which serves as a binding site for the methyl CpG–binding protein 2 (Mecp2) (Murgatroyd *et al.* 2009). Similarly, ELS leads to hypomethylation of a key regulatory region of the pituitary *Pomc* gene promoter, which similarly serves as a binding site for Mecp2 (Wu *et al.* 2014).

Altogether, studies in rodents and human suggest that early life trauma results in increased HPA axis responsiveness to stress in adulthood, partly due to epigenetic programming of genes involved in the stress regulation (e.g.; *Avp*, *Pomc*). Such epigenetic marks may confer an increased risk for the development of psychiatric disorders later in life. These insights might also help to identify opportunities to develop new strategies for diagnosis, prevention, and successful treatment of stress-related mental disorders.

1.3 Glucocorticoid Receptor (GR)

GCs are steroid hormones secreted by the adrenal glands. As mentioned above, GCs serve as a major mediator of the stress response. Since almost every cell type in the body is sensitive to GCs, these hormones regulate also other important processes in the body, such as metabolism, inflammation, immunity, and fetal development (Zanchi *et al.* 2010; Zunszain *et al.* 2011).

GCs exert their physiological effects through two types of receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). In the context of this thesis, I will focus only on GR.

1.3.1 Glucocorticoid receptor protein

GRs are ubiquitously expressed; however the levels of both mRNA and protein differ considerably between tissues (Turner *et al.* 2010). For example, *GR* can be found at high

levels in pituitary corticotropes and throughout the brain, especially in hippocampus and hypothalamic neurons (de Kloet *et al.* 1998).

The GR belongs to the superfamily of nuclear receptors that act in part as ligand-gated transcription factors. Similarly to other steroid receptors (mineralocorticoid receptor, MR; estrogen receptor ER; androgen receptor, AR), the GR is composed of 4 main domains (**Figure** 4):

- ✓ The N-terminal domain (NTD), containing the ligand-independent transactivation domain (AF-1), which is important for the interaction with other unrelated proteins necessary for the initiation of transcription;
- ✓ The DNA-binding domain (DBD), containing two zinc fingers, which controls GR binding to specific DNA sequences, termed glucocorticoid response elements (GRE). These palindromic motifs locate within the regulatory regions of GR target genes;
- ✓ The hinge region, containing a nuclear localization signal;
- ✓ The C-terminal ligand binding domain (LBD), responsible for the binding of GCs and ligand-induced activation of GR. The LBD contains also a second, ligand-dependent transactivation domain (AF-2), as well as sequences important for receptor dimerization, nuclear translocation, and interaction with co-activators (van der Laan *et al.* 2008; Nicolaides *et al.* 2010; Zanchi *et al.* 2010).

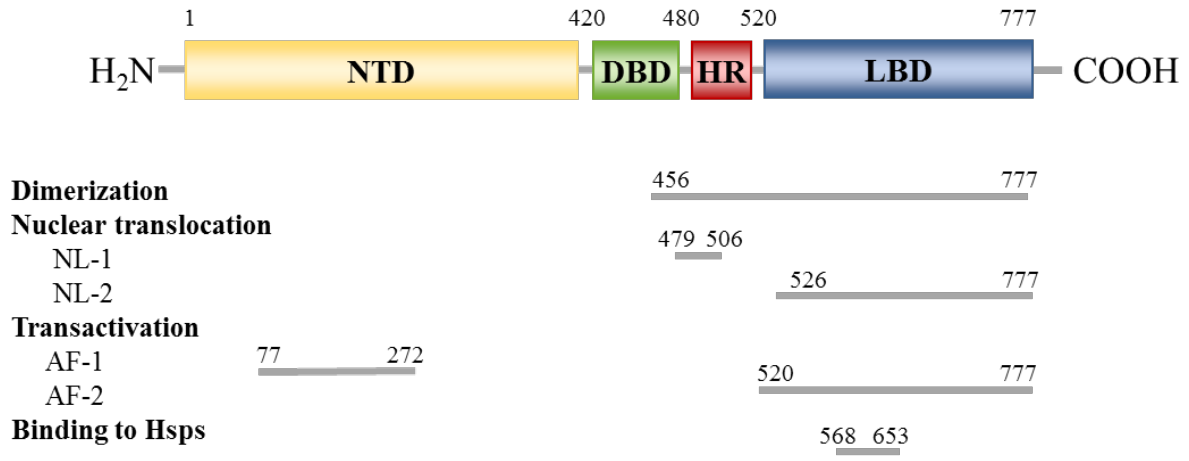

Figure 4: Schematic structure of human GR α
The GR protein is composed of distinct regions: N-terminal domain (NTD); DNA-binding domain (DBD); hinge region (HD); ligand-binding domain (LBD); activation function-1/2 (AF-1/2); nuclear localization signal 1/2 (NL1/2); and binding sites for heat shock proteins (Hsps). Numbers represent amino acid positions. Adapted from (Nicolaides *et al.* 2010)

1.3.2 Glucocorticoid receptor signaling

In the absence of ligand, GRs reside in the cytoplasm and are associated with a large complex of various proteins, including the chaperone heat-shock protein 90 (Hsp90) and the co-chaperone Fkbp5, which regulate folding, maturation and trafficking of the receptor (Grad *et al.* 2007*)*. Upon binding of the corticosteroid hormone, this complex undergoes conformational changes; hereby Fkbp5 is exchanged with Fkbp4, which then recruits dynein. This factor allows GR to translocate into the nucleus and to influence expression of its target genes by acting as a transcription factor (TF). GRs either directly bind to GREs as a homodimer or interact with other TFs as a monomer (Figure 5) (Binder *et al.* 2008; van der Laan *et al.* 2008). Typically, the GRE sequence consists of 15 nucleotides, containing a pair of inverted repeats and is separated by three nucleotides (n). The consensus sequence of the GRE is 5' AGAACAnnnTGTTCT 3', but many variations are possible (Del Monaco *et al.* 1997). The regulatory regions of GR target genes may contain one or more GREs, as well as imperfect GREs or half sites. Importantly, GREs have been identified for both, GR-inducible

and GR-repressed genes (positive and negative GREs) (Arteaga *et al.* 2008; Drouin *et al.* 1989; Hubler *et al.* 2004; Malkoski *et al.* 1999).

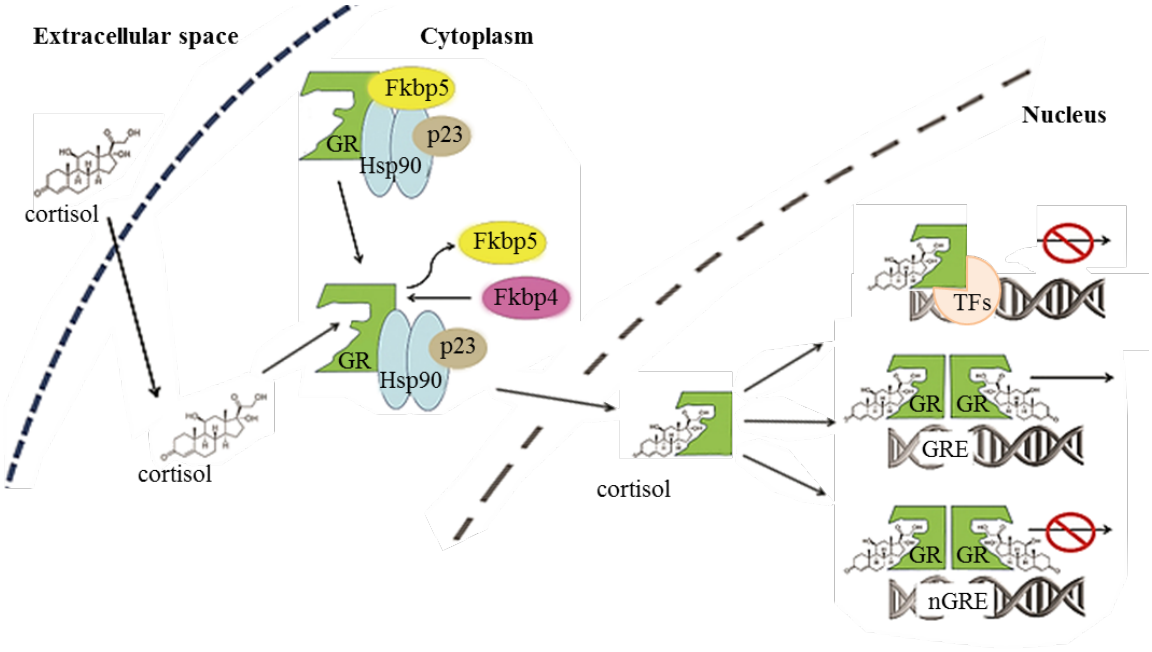

Figure 5: Molecular mechanism of GR-mediated signalling upon cortisol binding
In the unbound form the GR resides in the cytoplasm in a complex with Hsp90, p23, and Fkbp5. After ligand binding, Fkbp5 is replaced by Fkbp4. Subsequently Hsp90 and p23 detache and the GR translocates to the nucleus. Within the nucleus, the GR forms dimers or monomers and binds either to glucocorticoid response elements (GREs) or negative glucocorticoid response elements (nGREs) to activate or repress the transcription of target genes, respectively. GR monomers can also interact with other TFs to repress gene expression irrespectively of DNA binding. Adapted and modified from (van der Laan *et al.* 2008; Sinclair *et al.* 2013)

The expression of multiple GR target genes in neuronal tissues was recently investigated on a genome-wide scale (Sato *et al.* 2008; So *et al.* 2008; Datson *et al.* 2011). I will discuss here several examples of classical GR target genes, which were examined in this thesis. The most intensively studied gene is the *Fkbp5* gene encoding FK506 binding protein 51. Under resting conditions, *Fkbp5* is shown to be expressed ubiquitously in the adult mouse brain, with higher levels in hippocampus (Scharf *et al.* 2011). GCs have the ability to strongly increase its expression. For example, significant mRNA induction was found in the mouse PVN 4 and 8 h following dexamethasone application, which came back to baseline 24 h after injection. Changes were found also in response to stress, both after 30 minutes of restrained stress

(moderate stressor) and 24 h of food deprivation (strong and long lasting stressor). Thereby, induction of Fkbp5 was much stronger in tissues with low basal expression (e.g.; PVN, central amygdala) than in tissues with high basal expression (e.g.; hippocampus) (Scharf *et al.* 2011). It is important to note that Fkbp5 decreases the affinity of GR for its ligand; accordingly high levels of Fkbp5 expression can lead to glucocorticoid resistance. Thus GR-mediated induction of *Fkbp5* mRNA was suggested to correspond with GR sensitivity (Binder *et al.* 2008). The functional GRE (AGAACAgggTGTTCT) maps within intron E (between exons 5 and 6) and locates ~ 75 kb distal from the TSS. It is important to mention that this GRE is conserved between human, rat, and mouse. The functionality of this site was confirmed by different experiments, e.g.; gel shift assays, which demonstrated the interaction of the GR with the GRE at intron E, and mutational analysis, which showed abolished responsiveness to glucocorticoids. The authors predicted also another hormone binding site, which was shown to bind only progesterone receptor, but not GR (Hubler *et al*. 2004).

Another well-established GR target gene is *Sgk1* (serum- and GC-regulated serine/threonine protein kinase) (Sato *et al.* 2008), which is suggested to be involved in neuronal functions (e.g.; long-term memory formation) and the pathophysiology of different neurodegenerative brain diseases (e.g. Parkinson's and Alzheimer's disease) (Lang *et al.* 2010). The mouse and human *Sgk1* gene contain one known GRE, located ~ 1kb upstream of the TSS (So *et al.* 2007; Arteaga *et al.* 2008). *Sgk1* is described as an immediate early response gene that is rapidly induced by serum and GCs. Increasing doses of dex induce expression of *Sgk1* in rat hypothalamus. The highest peak of expression was seen 2 h after injection of 2mg/kg dex (Sato *et al.* 2008). In the rat hippocampus *Sgk1* mRNA was either strongly induced, as measured 60 min after corticosterone challenge (Sarabdjitsingh *et al.* 2010) or not regulated (van Gemert *et al.* 2006).

The best described function of another GR target gene *Dusp1* (Dual-specificity phosphatase 1) is inactivation of the mitogen-activated protein (MAP) kinase; therefore it may play an important role in cellular response to intrinsic and environmental signals. Within the *Dusp1* gene, several GREs has been predicted. These sites are homologues between human and mouse, and are located ~1.3, ~4.6, ~24, ~28, ~29 (~27 in mouse) kb upstream of the TSS. The study of Tchen *et al.* (2010) revealedthat in response to dex application, GR occupies its response elements 1.3, 4.6 and 24 in human, and 27 and 24 in mouse.

Gilz (glucocorticoid-induced leucine zipper) is widely expressed in various tissues, including brain. It is known to be strongly upregulated in response to GCs in different cell types, where *Gilz* mediates anti-inflammatory and immunosuppressive functions. There are multiple predicted GREs within the *Gilz* gene sequence. It was also demonstrated to be regulated by other steroid hormones (So *et al.* 2007).

Drr1 (Down-regulated in renal cell carcinoma 1; also known as *TU3A* and *Fam107A*) is mainly known for its role as a candidate tumor suppressor gene in the development of renal cell carcinoma (Ogawa *et al.* 1992). However, Schmidt *et al.*(2011), reported recently that *Drr1* is also expressed in the cerebellum and limbic areas of mouse brain and is significantly induced by stress (in pups by 24 h of maternal deprivation and in adult mice by 24 h of food deprivation) and dex (effect observed 8 h after injection). Increased *Drr1* mRNA was observed mainly in the PVN and hippocampal CA3 region. Additionally, *Drr1* induction was abolished by GR antagonist treatment. In the same study GR was shown to bind to all three predicted GREs, located within promoter region, intron 1 and 3'UTR.

Pdk4 (Pyruvate dehydrogenase kinase 4), similarly to *Sgk1*, is an immediate early response gene (Sato *et al.* 2008) and its activity contributes to the regulation of glucose metabolism. Expression of *Pdk4* mRNA is induced by glucocorticoid receptor binding to two distal GREs

(Connaughton *et al.* 2010) and has been shown to be elevated in the neonatal rodent hypothalamus after starvation (after 6 hours of maternal deprivation) (Ding *et al.* 2010).

1.3.3 Structure of the *Nr3c1* gene

The GR is encoded by the *Nr3c1* gene (nuclear receptor subfamily 3, group C, member 1), which is composed of 9 exons (Figure 6): 8 exons (2-9) comprising the GR protein sequence; and multiple untranslated first exons, responsible for the formation of various mRNA transcript variants, which encode however the same protein due to splicing to a common acceptor site within exon 2. Additional control to avoid translation of 5' heterogeneity is provided by an in-frame TGA stop codon located directly upstream of the ATG start codon (Turner *et al.* 2010). It is important to note, that the structure of the *GR* promoter is highly conserved between human, rat and mouse (Figure 6). For the human *GR* gene, at least 15 splice variants, derived from nine untranslated first exons have been published (Breslin *et al*. 2001; Presul *et al.* 2007; Turner *et al.* 2005), eleven for rat (McCormick *et al.* 2000) and ten for mouse (Bockmühl *et al.* 2011; Strähle *et al.* 1992). All identified alternative first exons are located either in distal (~32 kb upstream of the TSS) or proximal (~5 kb upstream of the TSS) promoter regions (Bockmühl *et al.* 2011; Breslin *et al*. 2001; Turner *et al.* 2005).

The generation of distinct mRNAs variants potentially influence mRNA processing, mRNA stability, and translation efficiency (Bockmühl *et al.* 2011). Although the *GR* is ubiquitously expressed, differential usage of distinct *GR* first exon promoters is shown to be responsible for its tissue- and cell-type specific expression (Cao-Lei *et al.* 2011; Turner *et al.* 2010). For example, human 1B and 1C transcripts are reported to be widely expressed in the brain, as well as in various peripheral tissues (Alt *et al.* 2010), (Turner *et al.* 2005). Other transcript variants show more restricted expression patterns: 1D is found mainly in testis, cerebellum, thymus (Presul *et al.* 2007) and hippocampus (Turner *et al.* 2005); 1A in thymocytes (Presul *et al.* 2007); 1E and 1F in the immune system and hippocampus (Turner *et al.* 2005). On the

other hand, rat 1.7 exon has been suggested to be specific to hippocampus; distally located 1.1 was detected in thymocytes; while 1.6 and 1.10 are the most abundantly expressed (McCormick *et al.* 2000). However, much less was known about the tissue distribution of mouse first exons.

Moreover, the activity of the different promoters might be affected by DNA methylation, as the proximal exons lie within a CpG island (Cao-Lei *et al.* 2011; Weaver *et al.* 2004) and contain in their sequence putative binding sites for multiple methylation- sensitive transcription factors, including Yin Yang 1 (YY1) (Breslin *et al.* 1998). Altogether, the complex regulation of *GR* expression allows each individual cell and tissue for appropriate response to environmental demands and tightly controlled *GR* levels, as even small changes may have significant pathophysiological consequences.

In addition to the heterogeneity of the *GR* 5'untranslated region (5'UTR), three different 3' splice variants can be generated (GR-α, GR-β, and GR-P), which differ in their functions. The predominant form GRα, which consist of 777 amino acids (aa) in human and 783 aa in mouse, is the most active form, which mediates GCs action. The last 50 aa of the C-terminus in GR-α are replaced by 15 unrelated aa in the splice variant GR-β, which are encoded by exon 9β. This form is unable to bind ligands due to changes in the LBD. GR-β contributes to ~ 1% of total GR and is suggested to act as an inhibitor of GR-α. A third variant, GR-P is translated to a truncated protein (676 aa), due to the lack of exons 8 and 9. GR-P has a disrupted LBD and is also unable to bind corticosteroids (Alt *et al.* 2010; Zanchi *et al.* 2010).

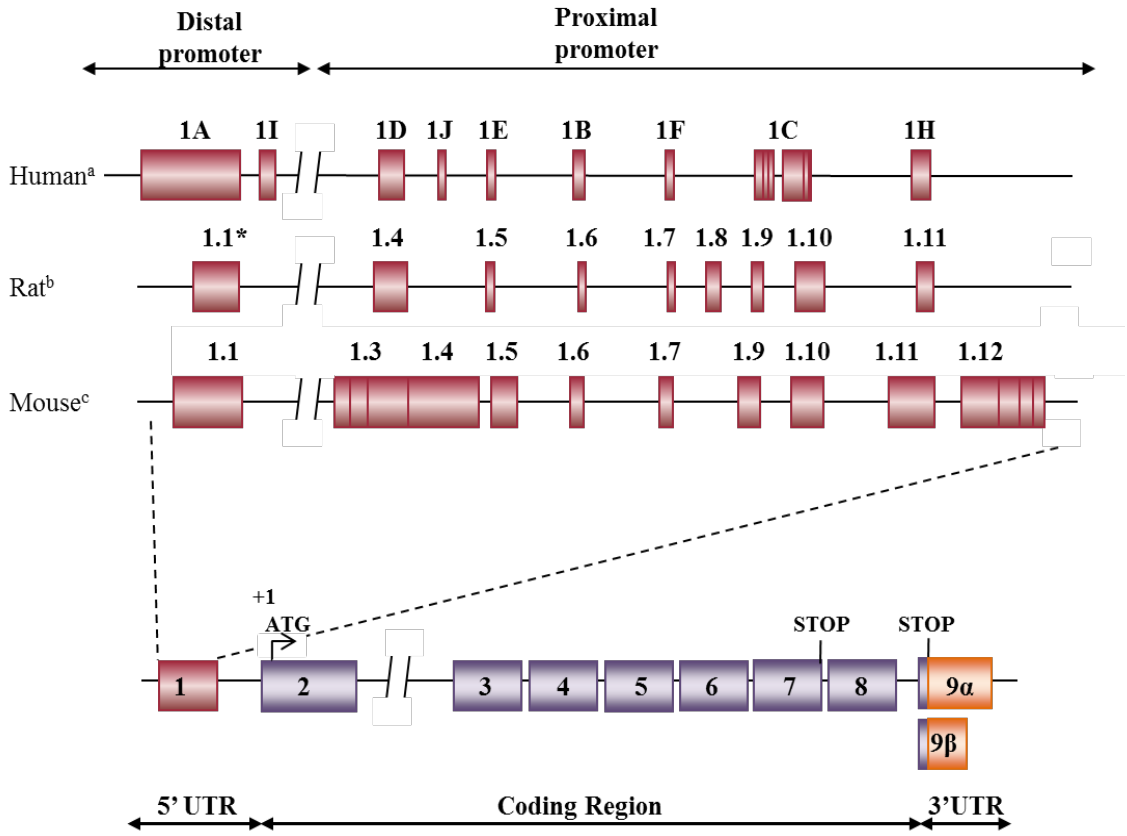

Figure 6: Schematic *Nr3c1* gene structure.
The upper part of the panel represents multiple untranslated first exons (in red), and their homology between human, rat, and mouse. Exons 1A and 1I in human; 1.1 in rat and mouse are located in the distal promoter (~32 kb upstream of the TSS), outside of the CpG island. Exons 1D-1H in human, 1.4-1.11 in rat, and 1.3-1.12 in mouse are located within the proximal GR promoter (~5 kb upstream of the TSS). The lower part of the panel represents exons 2-9 (in violet) which are transcribed to the GR protein. Three different splice variants can be formed: GR-α, containing exons 2-9α; GR-β, containing exons 2-9β; GR-P, containing exons 2-7. The 3'untranslated regions (3'UTR) are shown in orange. Note, the size of the exons is not shown on scale.
[a] (Breslin *et al.* 2001; Presul *et al.* 2007; Turner *et al.* 2005); [b] (McCormick *et al.* 2000),
[c] (Bockmühl *et al.* 2011; Strähle *et al.* 1992). Adopted and modified from (Bockmühl *et al.* 2011)

1.3.4 Epigenetic programming of *GR*

Accruing evidence supports a role of epigenetic programming in the regulation of glucocorticoid receptors. Most of the studies are focused on the methylation pattern upstream of rat exon 1.7 and its human orthologue 1F (see Figure 6), which contain a potential binding site for nerve growth factor- inducible protein A (NGFI-A, also called KROX, EGR1, or ZIF286). Indeed, the study of Weaver *et al.* (2004) identified a critical change in DNA methylation at the promoter region of the *GR* gene in response to variations in maternal care behaviour (in adult Long-Evans rat offspring of high- versus low LG-ABN mothers). The

authors have shown a reduced methylation of 5'CpGs at the binding site for the transcription factor NGFI-A at the promoter 1.7 in the hippocampus. This TF preferentially binds to unmethylated DNA and increases *GR* gene expression. Pups raised by high LG-ABN mothers show thus more efficient binding of NGFI-A, higher *GR* 1.7 transcript expression, and total GR protein in the hippocampus. In contrast, offspring receiving less maternal care, display higher CpG methylation, less NGFI-A binding and consequently reduced *GR* expression. These events correlated with decreased HPA-axis feedback efficiency and an increased emotional response to stress (Weaver *et al.* 2004). Subsequent human post-mortem studies identified site-specific increases in the methylation of human exon 1F in suicide completers with a history of childhood abuse (in comparison to suicide victims with no history of abuse and control subjects). These changes were accompanied by altered NGFI-A binding, and decreased expression of the *GR* 1F transcript as well as total GR protein (McGowan *et al.* 2009). It is important to note that epigenetic changes caused by early life stress do not appear to be restricted to brain tissues, but can be also detected in peripheral tissues, e.g.; a history of childhood adversity is associated with increased cytosine methylation at the NGFI-A binding site at exon 1F in leukocytes of healthy adults and associates with an attenuated cortisol response to the dex/CRH test (Tyrka *et al.* 2012). Similarly, subtle increases in the methylation at this specific site in response to prenatal stress was measured in umbilical cord blood of newborns (Oberlander *et al.* 2008) and blood cells of adolescent children (Radtke *et al.* 2011). These findings suggest that early-life stress may result in epigenetic marking of specific CpGs within the *GR* promoter, which lead to changes in glucocorticoid receptor transcription in humans and rats, and consequently altered regulation of HPA-axis activity and stress responsiveness.

The existence of additional binding sites for various TFs located within the CpG island suggests the potential control of *GR* expression by other promoters than 1.7 (1F) in a

methylation-dependent manner. In fact, some findings reported changes in expression and/or methylation of additional alternative *GR* first exons. Labonte *et al.* (2012) observed, in hippocampal tissues of suicide victims with a history of ELS, differential methylation within exons 1B, 1C, and 1H and their reduced transcription. These changes contributed to a decreased level of total *GR* mRNA (Labonte *et al.* 2012). Hypomethylation of the 1.10 promoter and an increased total level of *GR* was also observed in the liver of offspring of Wistar rats fed with protein-restricted diet during pregnancy (Lillycrop *et al.* 2007). Recently, McGowan *et al* (2009), reported that among the offspring of high LG-ABN mothers, in addition to the previously described elevated levels of the 1.7 splice variant, also others ones known to be expressed in the hippocampus (i.e.; 1.5, 1.6, 1.10, and 1.11) showed an increased transcription. These may suggest that *Nr3c1* gene regulation is based on the coordinated regulation of various splice variants, rather than single ones (McGowan *et al.* 2011).

Recently, our studies have shown that ELS leads to up-regulation of the *GR* mRNA in the hypothalamic PVN of adult male mice with a history of ELS. This finding is correlated with an increased site-specific DNA methylation of a single CpG dinucleotide (termed here CpG3) located upstream of the CGI, within the so-called "CpG island shore". Importantly, ELS-mediated epigenetic programming of *GR* appears to be highly tissue-specific and is observed exclusively in the hypothalamic PVN, but not at other sites involved in GR-mediated negative feedback, e.g.; hippocampus and pituitary (Bockmühl *et al.* submitted).

Computational analysis predicted the residue CpG3 to be part of a binding site for the ubiquitously expressed zinc finger transcription factor Yin Yang 1 (YY1) (Bockmühl *et al.* submitted). The name Yin Yang 1 reflects the multifunctional nature of this factor to act as a transcriptional activator, repressor, or initiator of transcription, depending on cellular context and the availability of additional regulatory factors (Shi, Lee, and Galvin 1997). The predicted binding site at the antisense strand (5'-CGCCATCTT-3'), strongly resembles the consensus

sequence 5'-(C/g/a)(G/t)(C/t/a)CATN(T/a)(T/g/c)-3' (Hyde-DeRuyscher, Jennings, and Shenk 1995) and is homologues to a previously identified distal YY1 binding site (dYY1) within the human *GR* (Breslin *et al.* 1998). By a series of *in vitro* and *in vivo* studies, we showed YY1 to occupy its distal binding site within the mouse *GR* promoter. As YY1 binding is methylation-sensitive (Kim *et al.* 2003), methylation of the CpG3 site in the PVN of ELS mice abolishes its binding and allows for enhanced *GR* promoter activity, resulting in up-regulated *GR* mRNA (Bockmühl *et. al*. submitted).

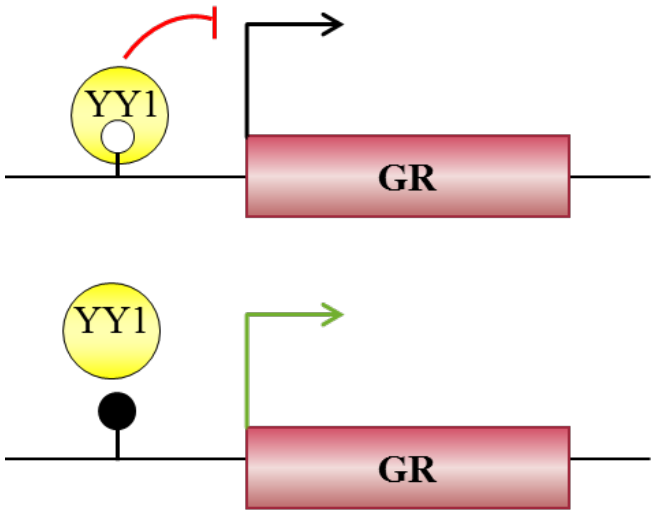

Figure 7: Summary of finding Y. Bockmühl *et al.* submitted
In hypothalamic PVN, site-specific CpG methylation, due to maternal separation during the first 10 days of life, prev:ents binding of the transcriptional repressor YY1, thus allowing for enhanced GR promoter activity, resulting in elevated GR protein levels. On the other hand, control mice exhibit lower methylation levels. As a result, YY1 can bind and repress *GR* promoter activity.

1.4 Aim of the thesis

Numerous studies in human have shown that adverse events experienced in early life are important risk factors for the development of stress-related psychiatric disorders, including anxiety and MDD. Previous work from our laboratory has shown that male C57BL/6N mice exposed to early-life stress (ELS) display distinct neuroendocrine and behavioural abnormalities in adulthood important to MDD. This phenotype is accompanied by enduring epigenetic programming of genes involved in hypothalamic-pituitary adrenal (HPA) axis regulation: arginine vasopressin (*Avp*) (Murgatroyd *et al.* 2009) and pro-opiomelanocortin (*Pomc*) (Y. Wu *et al.* 2014). These results raised the question, whether the *Nr3c1* gene, which encodes the glucocorticoid receptor (a major negative feedback regulator of the stress axis), is also epigenetically programmed by ELS in mice.

The aims of the work presented here were:

- ✓ to investigate the tissue distribution of newly identified *GR* alternative first exons and their regulation by different signaling pathways in ELS-naïve mice [work done jointly with Yvonne Bockmühl (Bockmühl *et al.* 2011)];
- ✓ to investigate regulation of *GR* first exons by ELS [work done jointly with Yvonne Bockmühl (Bockmühl *et al.* submitted];
- ✓ to assess the biological activity of enhanced GR expression in the PVN, by measuring the transcriptional regulation of classical downstream GR target gene;
- ✓ to analyse the methylation pattern of a YY1 DNA binding site located within the CpG island shore region of the *GR* promoter in CD1 mice and human tissues.

CHAPTER 2 MATERIAL AND METHODS

2.1 **Animals**

C57BL/6N and CD1 mice were housed under standard laboratory conditions, with 12-h daily illumination (lights on at 6:00) at the animal facility of the Max-Planck Institute of Psychiatry. Time-pregnant C57BL/6N females were purchased from Charles River, Sulzfeld, Germany. All procedures were approved by the Regierung von Oberbayern and were in accordance with European Union Directive 86/609/EEC

2.1.1 Maternal separation procedure [1]

The maternal separation (MS) procedure was used to induce early-life stress (ELS) in C57BL/6N mice. The day of pup's birth was considered as postnatal day 0 (PND 0). In the ELS group, pups were physically separated from their mothers (3 h daily, starting from PND 1 to PND 10) by placing them, as individual litters, to a clean temperature-controlled (heating pad) cage. After 3 hours of separation, pups were transferred back in the maternal nest. In the control group, animals were left undisturbed and remained in their home cages during the whole experiment. In both experimental groups, pups were kept with their mothers until weaning (PND 21) and were thereafter housed in sex-matched groups (~5 mice per cage). Only males were used for the following experiments (DNA methylation and mRNA expression analyses, *in vivo* ChIP experiments, and endocrine analysis).

2.1.2 Corticosterone treatment [2]

The first cohort (used for quantitative RT-PCR) of 10 weeks old male mice (control and ELS) was injected intraperitoneally at 8 a.m. either with saline with cyclodextrin (vehicle) or

[1] ELS was performed by employees of the animal facilities of the MPI of Psychiatry, under the supervision of Dr. Alexandre Patchev and Prof. Dr. Osborne Almeida

[2] CORT injection was performed by Dr. Alexandre Patchev

corticosterone complexed in 25% cyclodextrin (3 different doses: 0.1; 1.0; 10.0 mg per kg of body weight). Animals were sacrificed 3 h after injection. The second cohort (used for ISH and ChIP) was injected intraperitoneally either with saline or only one dose of corticosterone (1.0 mg per kg of body weight). Animals were sacrificed 90 min after injection. Brains from both cohorts were collected and preceded as described in the next section.

2.1.3 Tissue preparation

Male C57BL/6N mice were sacrificed at three time points (10 days, 6 weeks, and 3 months) by cervical dislocation. Brain tissues were collected and immediately snap-frozen by immersion in cold iso-pentane and stored at -80°C. For IHC assay brains of 3 month old mice were fixed with PFA through cardiac perfusion, fixed in 4% PFA overnight, sucrose cryopreserved (15 % and 30%), snap-frozen by immersion in cold iso-pentane and stored in -80°C. Brains were next cryosectioned (10 μm thickness) and stained with cresyl violet to verify anatomical precision. Paxinos mouse stereotaxic brain atlas was used as a reference for histological control (Franklin and Paxinos 2008). Sections containing the region of interest (PVN: bregma –0.75 to –0.85; hippocampus: bregma −1,70 to −1,90) were mounted on Superfrost glass slides, stored at -20°C and used for immunohistochemistry and *in situ* hybridisation. In addition, tissue punches (0.8 mm diameter) were taken from brain areas containing PVN and dentate gyrus and frozen at -80°C for further analysis.

2.2 Human samples

2.2.1 Collection of peripheral blood cells and buccal swabs

Human peripheral blood cells and buccal swabs were obtained from healthy male volunteers, age 27-60 years for subsequent DNA extraction and methylation analysis.

2.2.2 Post mortem brain samples [3]

Human post mortem brain samples (inferior cortex, hippocampus, and hypothalamus) were gifted by the Brain Bank Center Munich (Zentrum für Neuropathologie und Prionforschung). For our analysis we obtained tissues from males aged 35-65 years (the cause of death was not related to a brain disease). Appropriate areas of interest were identified by histological control (staining with cresyl violet), punched, and frozen at -80°C for further bisulphite sequencing analysis.

2.3 RNA analysis

2.3.1 RNA isolation

Total RNA from snap frozen brain tissues was isolated using a previously described protocol for simultaneously extracting DNA and RNA from tiny neuroanatomically-defined brain regions (Bettscheider, Murgatroyd, and Spengler 2011). Briefly, samples were homogenised at RT by vortexing in 400 µl of guanidinium thiocyanate (GTC) buffer (4.5 M guanidinium thiocyanate; 2% N-Lauroylsarcosine; 50 mM EDTA pH 8; 25 mM Tris-HCl pH 7.5; 0.1 M beta-mercaptoethanol; 0.2% antifoam A) and passing several times through a syringe needle. Each sample was then splited into two equal parts (200 µl each), the first one for RNA and the second one for DNA extraction. The total volume of the homogenate was used only when processing with one type of nucleic acid was required. For RNA purification, 1/10 volume of NaOAc, 1 volume of acidic phenol and 1/2 volume of chloroform:isoamyl (24:1) was added. Each sample was vortexted, incubated on ice for 10 min and centrifuged for 20 min at 13.000 rpm at 4 °C. The RNA in upper aqueous phase was then transferred to a new tube, precipitated with an equal volume of 70% EtOH and further processed according to the manufacturer's instructions (Nucleospin RNA II kit, Macherey Nagel). RNA was eluted in 25

[3] The appropriate areas were dissected out under the guidance of Dr. med. Thomas Arzberger

µl of RNase-free water and the concentration was measured spectrophotometrically with a NanoDrop photometer.

2.3.2 cDNA synthesis (reverse transcription)

For cDNA synthesis, 200 ng of RNA was reverse-transcribed using the RevertAid Premium ReverseTranscriptase (Fermentas) and oligo(dT) primers. The reaction was performed according to the manufacturer's instructions.

2.3.3 Quantitative PCR analysis

Expression levels of genes of interest were determined by using the LightCycler 2.0 Instrument (Roche, Mannheim, Germany) and LightCycler FastStart DNA Master plus SYBR GreenI Kit (Roche). DNA amounts precipitated during chromatin immunoprecipitation were analysed using the MJ Mini Opticon light cycler (Bio-Rad) and the Absolute Blue QPCR Sybr green mix (ABgene). All experiments were performed according to the manufacturer's protocols. Threshold and noise band were set in all compared runs to the same level. qPCR results were determined by the $2^{-\Delta Ct}$ method (Livak *et al.* 2001) using the PCR efficiency. Relative expression of analysed genes was normalized to the expression of the house keeping gene *Atp5j*. Fold enrichment of ChIP samples was normalized relative to values of control samples. The primers used for qRT-PCR are listed in Table 1 and 3.

2.4 DNA analysis

2.4.1 Genomic DNA isolation

For genomic DNA purification from the snap frozen brain tissues, the second half of the homogenate (as described in section 2.3.1 "RNA isolation") was used. To each tube equal volumes of lysis (AL) buffer and 100% EtOH were added and loaded on a Spin Column

(DNeasy Blood and Tissue Kit, Qiagen). Washing steps were performed according to the manufacturer's protocol and the DNA was eluted with 70°C warm elution (AE) buffer. Genomic DNA from human peripheral blood and buccal swabs was extracted using the QIAmp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.4.2 PCR reaction /Colony-PCR

Standard PCR reactions were performed using the Taq DNA Polymerase Kit (Fermentas). The PCR reaction mix was prepared in a total volume of 25 µl in the presence of 2 µl of DNA template, 1x *Taq* Buffer with (NH4)2SO4; 2.5-3.0 mM MgCl2; 0.6 mM dNTP; 0.4 µM forward and reverse primer and 0.04 u/µl Taq polymerase final concentration. Standard amplification conditions are:

1 cycle	6 min 95°C
45–50 cycle	1 min 95 °C, 1 min at optimal annealing temperature, 1 min 72 °C
1 cycle	5 min 72 °C

For colony PCR a small amount of a bacterial colony was picked from an agar plate and added to the PCR reaction mixture containing T7- and SP6-primers (Sigma, Steinheim, Germany). Clones were amplified using the following conditions:

1 cycle	4 min at 95°C
10 cycles	30 s at 94°C, 30 s at 56°C and 30 s at 72°C
30 cycles	30 s at 94°C, 30 s at 48°C and 30 s at 72°C
1 cycle	5 min at 72° C

2.4.3 Bisulfite sequencing

Sodium bisulfite conversion was performed with 400 ng of genomic DNA using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. This chemical reaction leads to the conversion of non-methylated cytosines to uracils. After PCR amplification with primers specific to bisulfite converted DNA, products were purified (NucleoFast PCR Cleanup Plate, Macherey-Nagel) and ligated overnight at 4°C into pGEM-T vectors. Purified ligation products were used for transformation with electrocompetent DH5α bacteria. After overnight incubation at 37°C with blue/white selection, positive clones were amplified. Colony PCR reactions containing the expected amplicons were cleaned up using a commercially available kit (Machery Nagel Nucleofast) and subjected to the Big-Dye sequencing reaction.

2.4.4 Big Dye terminator reaction and sequencing

3 µl/well of master mix for Big-Dye reaction: 0.2 µM sequencing primer (usually T7, but also SP6 or T3), 1x Sequencing Buffer and Ready Reaction Mix of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) was dispensed into a 96-well plate and mixed with 2 µl of cleaned up colony PCR product or 0.5-2.0 µl purified vector. The reaction was proceeded on a thermocycler with the following parameters:

1 cycle	1 min at 96°C
35 cycles	10 s at 96°C, 5 s at 50°C and 4 min at 60°C

After a clean-up step (Millipore Montage 96 Sequencing Clean-Up Kit) products were processed on a capillary sequencer (ABI 3100 DNA). For bisulfite sequencing ~24 independent recombinant clones per amplicon were analysed.

2.4.5 Chromatin immunoprecipitation (ChIP) assay

In vivo chromatin immunoprecipitation assays were performed as described previously (Murgatroyd *et al.* 2009). Frozen tissue punches were resuspended into 150 µl of ice-cold PBS supplemented with 1x protease inhibitor cocktail (PIC) (Sigma Aldrich) and then passed several times through a syringe needle. After a centrifugation step (4.000 rpm/ 5 min/ 4°C), 150 µl of PBS containing PIC was added to the pellet. Samples were then incubated for 15 min at RT on a rotation platform in the presence of formaldehyde (final concentration 1%) to cross-link proteins with DNA. The reaction was stopped by adding glycine (final concentration 125 mM) and samples were incubated with rotation for additional 5 min. Samples were washed twice with 150 µl PBS containing PIC by successive centrifugation steps (4000 rpm/ 5 min/ 4°C) and the pellet was resuspended in cell lysis buffer (with protease inhibitor). After incubation for 10 minutes on ice samples were resuspended in nuclear lysis buffer and sonicated 3 times for 3-4 min (30 seconds "ON" / 30 seconds "OFF") with the Bioruptor TM (Diagenode, Liege, Belgium) in an ice-water bath. The DNA concentration in the sheared chromatin was determined with a SmartSpec Plus spectrophotometer (Bio-Rad) and aliquots (OD_{260nm} = 0.8) were then subjected to immunoprecipitation using an antibody specific to GR (rabbit polyclonal anti-GR H300X in dilution 1:100; Santa Cruz). To reduce unspecific binding during immunoprecipitation, antibodies were pre-incubated with sheared chromatin DNA on a rotating platform at 12°C overnight, while Dynabeads Protein G (Invitrogen) were added on the following day and incubated for another hour at 12°C on a rotating platform. Washing of the Protein G bead-antibody/chromatin complex was performed as described in the Upstate Magna ChIP G protocol, but for 7-10 min per wash-buffer. After reversecrosslinking, protein digestion, and DNA isolation using the UltraClean PCR Clean-Up Kit (Mobio), fragments were dissolved in 100 µl TE buffer and subjected to real-time PCR.

2.4.6 Ligation

Purified PCR products were ligated overnight at 4°C into pGEM-T vector using the pGEM-T Easy Vector System (Promega) according to the manufacturer's protocol. After ligation, products were precipitated by adding 1 µl glycogen, 0.1 volume NaAc (3M) and 2.5 volumes of pure ethanol to the ligation mix and placed in liquid nitrogen for 1 min. Samples were then centrifuged (4°C, 12.000 rpm, 20 min), and the pellet was washed with 70% ethanol prior to another centrifugation step (4°C, 12.000 rpm, 10 min), air dried and dissolved in 5 µl di-distilled water (ddH_2O).

2.4.7 Transformation into electrocompetent bacteria

Aliquots (40 µl) of electrocompetent DH5α bacteria were thawed on ice and diluted in 100 µl of ice-cold water. 45 µl of diluted bacteria was mixed with 5 µl of cleaned up ligation product or 0.5 µl plasmid-DNA (~1 µg/µl) and incubated 1 min on ice. The mixture was then transferred to a pre-cooled Gene Pulser Cuvette (width 0.1 cm; Biorad) and electrotransformed (settings were 1.5 kV, 200Ω, 25 µF). Immediately after electroporation, 1 ml of SOB medium (20 g/l tryptone; 5 g/l yeast extract; 10 mM NaCl; 2.5 mM KCl) was added to the bacteria and incubated for 1 h at 37°C. 50-200 µl of the suspension was spread out on an agar plate containing ampicillin (50 µg/ml) and coated with 200 µl of indicator solution [a mixture of 105 µl ddH_2O, 40 µl DMSO, 30 µl IPTG (0.1 M) and 25 µl X-gal (20 mg/ml)] and incubated overnight at 37°C.

2.4.8 Blue/white screening

The pGEM-T vector contains multiple cloning sites within the *LacZ* gene coding for β-galactosidase. The functional enzyme is activated in the presence of IPTG (isopropyl-[beta]-D-thiogalactopyranoside) and converts the substrate X-Gal (5-bromo-4-chloro-3-indolyl-

[beta]-D-galactopyranoside) into a bright blue product. Successful cloning of an insert disrupts the coding sequence of *LacZ*. Thus, bacteria harbouring the recombinant plasmid form white colonies. In the absence of an insert, bacterial colonies stain blue. Colony PCR from single positive clones was conducted and the expected size of inserts was verified by agarose gel electrophoresis.

2.5 Plasmid preparations

2.5.1 Generation of plasmid constructs used as standards for qRT-PCR

pGl3-Control-T7: Two phosphorylated oligonucleotides containing the T7 promoter sequence (see Annex I) were annealed to generate an oligonucleotide with a 5'-*HindIII* and 3'-NcoI overhang and a nested *HindIII* restriction site at the 3'end (the 5'-HindIII overhang is not reconstituted after ligation). The annealed oligonucleotide was cloned into a *HindIII/NcoI* digested pGl3-Control vector (Promega).

pGl3-Control-T7-exon 1 vectors: The DNA fragments coding for alternative *GR*-exon first variants were PCR amplified from cDNA using forward primers specific for each transcript and containing a *HindIII* restriction site, with the a common reverse primer directed against exon 2 containing an *NcoI* restriction site (Ex2R-NcoI). Primers used for this reaction are listed in Table 2. The PCR product was cloned into pGl3-Control-T7 vector and its correct sequence was verified by sequencing.

pCR2.1-exon2: The sequence for *GR* exon 2 containing the target region for qRT-PCR reverse primer (Table 1) was PCR amplified using Ex2 primers (Tabble 2). PCR product was cloned into pCR2.1 vector (Invitrogen), and verified by sequencing.

pCR2.1-exon2-1: pCR2.1-exon2 was digested by *HindIII* and *SspI* restriction enzymes and ligated with *HindIII-SspI* exon 1-fragments derived from pGl3-Control-T7-exon 1 vectors. The cloning product pCR2.1-exon2-1 serves as external standard for exon 1 transcript

quantification. qRT- PCR standard for total *GR* was obtained by TOPO TA Cloning the PCR product that was amplified by total *GR* primers (Table 1) into pCR2.1 vector.

2.5.2 Plasmid DNA: mini-preparations

A single growing bacterial colony was picked from an agar plate and incubated in 2.0 ml SOB medium (20 g/l tryptone; 5 g/l yeast extract; 10 mM NaCl; 2.5 mM KCl) supplemented with ampicillin (200 µg/ml). A culture was grown overnight at 37°C with vigorous shaking. The suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged (14.000 rpm/ 1 min/ RT) to pellet the bacteria. Plasmid DNA was isolated using the peqGOLD Plasmid Miniprep Kit I (Peqlab) according to the manufacturer's instructions. To identify positive clones 0.5 µl of purified plasmids was used for restriction analysis and additional 0.5 µl for sequencing.

2.5.3 Plasmid DNA: maxi-preparations

An aliquot (2 ml) of an overnight bacterial preculture was used to inoculate the main culture containing 40 ml of TB medium (12 g/l tryptone; 24 g/l yeast extract; 0.4% (v/v) glycerol; 2.3 g/l KH2PO4; 16.4 g/l K2HPO4·3H2O) supplemented with ampicillin (200 µg/ml) in a 500 ml flask After overnight incubation at 37°C with vigorous shaking plasmid DNA was isolated and purified according to the NucleoBond PC100 (Machery Nagel, Düren, Germany) kit according to the manufacturer's protocol. After precipitation of the eluate with isopropanol, the pellet was dissolved in 400 µl ddH_2O and further purified by a phenol/chloroform extraction.

2.5.4 Phenol/chloroform extraction of plasmid DNA

One volume of TE-saturated phenol/chloroform/isoamylalcohol (25:24:1) (pH 8.0) was added to the DNA solution. Samples were vortexed for 30 seconds and then centrifuged at 14.000

rpm for 1 minute. The aqueous phase (upper layer) was transferred into a new 1.5 ml tube, mixed with one volume of chloroform/isoamylalcohol (24:1) and centrifuged again. The DNA from the aqueous phase (upper layer) was transferred into a new 1.5 ml tube and precipitated. The pellet was dissolved in 40-200 µl TE buffer. The DNA concentration was determined with the Smart spec Plus Spectrophotometer (Biorad).

2.5.5 Restriction digest of plasmid DNA

0.2 to 25 µg plasmid DNA were cleaved by restriction endonucleases (Fermentas, St. Leon-Rot, Germany or New England Biolabs, Frankfurt am Main, Germany) at selected restriction sites in 10 µl to 100 µl reaction volume to obtain specific DNA fragments. Buffers were chosen according to manufacturer's recommendations. Vector backbones were phosphorylated when necessary. Reactions were carried out at the recommended temperature and incubations lasted 1-16 h.

2.5.6 Blunting of vectors and inserts

3-5 µg of linearized vectors or inserts were blunted by adding 2 µl dNTPs (2 mM) and 1 µl T4 DNA Polymerase (Fermentas) to a total volume of 50 µl. The reaction was incubated for 5 min at RT and inactivated for 20 min at 70°C.

2.5.7 Absolute quantification of exon 1 transcripts

Vectors containing fragments coding for alternative *GR*-exon first variants were linearized by *HindIII* (Thermo scientific) digestion and the DNA concentration was measured with a NanoPhotometer. Finally the plasmids were serially diluted (1:10) to serve as an external homologous DNA standard of known copy numbers. Plasmid concentrations of 10^{-5} to 10^{-10} µg/µl were used, within the range of the cDNA samples, to provide calibration curves for

each exon first transcript. Copy numbers of each exon 1 transcript were extrapolated from the calibration curve. Molecules per gram plasmid standard were calculated using the formula: NA x MW-1; NA = Avogadro constant, MW = molecular weight of plasmid.

2.6 Immunohistochemistry

Frozen brain sections were allowed to warm-up slowly at RT for 30 minutes, fixed with pre-warmed 4% paraformaldehyde in PBS containing 2% sucrose for 5 minutes at 37°C and rinsed with PBS. Sections were incubated for 2 hours with blocking solution (5% BSA, 5% normal goat serum, 0.05% Triton X-100, 50 mM Tris-HCl, 50 mM NaCl, pH 7.4); next incubated with the primary antibodies diluted in blocking solution in a humidifying chamber for 16 h at 12°C. Sections were then washed three times with PBS for 10 minutes and incubated at RT for 2 h with secondary antibodies diluted in blocking solution (1/1000). Sections were washed with PBS three times for 10 minutes prior to addition of 4,6-Diamidin-2-phenylindol (DAPI) diluted in PBS (1/6000) for 5 minutes. Finally, sections were washed with PBS three times for 10 minutes before mounting in Mowiol on SuperFrost microscope slides. Confocal images were taken using an Olympus laser scanning confocal microscope.

2.7 Cell culture

SK-N-MC (ATCC no. HTB-10), Be(2)-M17(ATCC no. CRL-226), SHS-Y5Y (ATCC no. CRL-226) , AtT-20 (ATCC no.CCL-89), N1E-115, HW3.5 (Baj *et al.* 2005) and N6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, catalogue no. 41965-062, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin. Cells were seeded onto cell culture dishes, one day prior to any manipulation, at densities of approximately 1-2 x 10^4 cells per cm^2.

2.7.1 bFGF treatment

AtT-20 cells were maintained in DMEM without phenol red (catalogue no. 31053-044, Invitrogen) containing 10% charcoal-treated serum before treatment with bFGF (20 ng/ml; catalogue no. 450-33 Peprotech, Hamburg, Germany) for 24 h. Serum was depleted of steroids by overnight incubation with active charcoal and subsequent filtration.

2.7.2 Potassium-induced depolarization

Depolarization experiments in N6 cells were carried out by adding KCl at a final concentration of 50 mM to the medium and incubated for 30 min or 2 h.

2.8 Statistical analysis

Statistical analyses were performed by using t-test or two- way ANOVA, and Newman–Keuls test as *post hoc* test, if appropriate. Data are presented as mean ± SEM. Significance levels were set at $p<0.05$ and a trend was recognized at $p<0.1$. For calculations SigmaPlot software was used.

2.9 Primer sequences and antibodies

Table 1: Expression analysis

Gene/ transcript	Sequence (5'- 3')	Exons	Product size [bp]	Annealing Temp. [°C]
Total *GR*	fwd: caagggtctggagaggacaa rev: tacagcttccacacgtcagc	2/3	220	61
exon1.1	fwd: gttcccatcgcagcctgatcat	1	238	63
exon1.3	fwd: gtggcgtcactgctctttacca	1	229	60
exon1.4c	fwd: aaggagaggtcaggagtttgg	1	173	61
exon1.5	fwd: cagctcgctaagagggttttgcattc	1	198	61
exon1.6	fwd: gacctggcagcacgcgagtc	1	188	64
exon1.7	fwd: ccagggagaagagaaactaaagaaact	1	200	64
exon1.9	fwd: cggagtcagtgcctggag	1	209	61
exon1.10	fwd: ggcgactgttgacttccttc rev: ggagctaaggattctttggag	1/2	156	61
exon1.11	fwd: ggctcgctgcctgcagcccagacttc	1	229	61
exon1.12	fwd: gaattgcggccttaccact	1	186	63
exon 2	rev: gtgaagacgcagaaaccttgactgtag	2		60-64
Atp5j	fwd: tattggcccagagtatcagca rev: ggggtttgtcgatgacttcaaat	3/4	134	60
Uros	fwd: tctgaaagacagatggaatgcc rev: ccacacggaaagagaagaggc	3/5,6	174	60
Avp	fwd: tcgccaggatgctcaacac rev: ttggtccgaagcagcgtc	1/2	173	56
Fkbp5	fwd: gggtgtacgccaacatgttc rev: gaggagggccgagttcatt	13/14	199	63
Sgk1	fwd: cttctggactaatgatgatgcagc rev: tgccctttccgatcactttc	2/3	268	59
Dusp1	fwd: gagctgtgcagcaaacagtc rev: cttccgagaagcgtgatagg	2/3	177	62
Gilz	fwd: gataacagtgcctccggag rev: gggatggaggagtgaagac	1/2	246	62
Drr1 (Fam107a)	fwd: caccgtgagctgcttatgaac rev: tcccggactttgatgaactc	3/5	245	60
Pdk4	fwd: cctttggctggttttggtta rev: cctgcttgggatacaccagt	9,10/11	255	64

Table 2: Primers for cloning

Gene/ transcript	Sequence (5'- 3')	Comment
HindIII-1.1	fwd: agagaagcttcctctcgtgcatgatgcaacacctg	primer to amplify exon 1.1
HindIII -1.3	fwd: agagaagcttaaagagcacctctgccaaaatgg	primer to amplify exon 1.3
HindIII-1.4c	fwd: d agagaagcttcctcagctccgatcagaagtg	primer to amplify exon 1.4c
HindIII-1.5	fwd: agagaagcttcaacttactattccgtctgcaacttg	primer to amplify exon 1.5
HindIII-1.7	fwd: agagaagcttccgagcggttccaagccg	primer to amplify exon 1.7
HindIII-1.9	fwd: agagaagcttccctcgctcggtcccgctcg	primer to amplify exon 1.9
HindIII-1.10	fwd: agagaagcttagaacgcgcgcggggagacg	primer to amplify exon 1.10
HindIII-1.11	fwd: agagaagcttatccctggcccagcgcgctgc	primer to amplify exon 1.11
HindIII-1.12a	fwd: agagaagcttacatttctcccttcacctcgcac	primer to amplify exon 1.12a
Ex2R-*NcoI*	rev: gactccaaagaatccttagctcccatggagag	Common primer to amplify exon 1
Ex2	fwd: actaatcggatcagagataatgtgg rev: agaggatcctctgctgcttggaatctg	
T7	s: agctaatacgactcactatagggaagcttc as: catggaagcttccctatagtgagtcgtatt	

Table 3: Methylation analysis

Gene/ transcript	Sequence (5'- 3')	Product size [bp]	Annealing Temp. [°C]
Mouse *GR* (CpG1-9)	fwd: ttttttttttggagaaggaggtt rev: taaaaaatataaaaactacccaatatatc	193	51
Human *GR* (CpG1-11)	fwd: ttagttgatttgtgttaagggat rev: ataaactttcaacaaacctctta	257	54

Table 4: ChIP assay

Gene/ transcript	Sequence (5'- 3')	Product size [bp]	Annealing Temp. [°C]
Fkbp5	fwd: gttcagctgtgcaatccaga rev: agggtgttctgtgctcttcaa	134	62
Sgk1	fwd: acctcctcacgtgttcttgg rev: ggtatgaggggtcaggaat	187	63
Dusp1 (-27 kb)	fwd: agaagagacccactcaggcc rev: ctgggtccactttcccacta	109	62

Table 5: Antibodies

Antibody	Application (dilution)	Company
GR (H300 X)	ChIP (1:100) IHC (1:1000)	SantaCruz Biotechnology
Avp (PS 41)	IHC (1:100)	H. Gainer (Ben-Barak *et al.* 1985)
Crh	IHC (5:100)	Bachem, Torrance, CA, USA

Primary antibodies were visualized using the appropriate secondary antibodies conjugated to Dylight 488, Dylight 594 (1:500, Diagenode, 715-485-150, 711-585-152), Alexa 647 (1:500, Dianova, Hamburg, Germany, 706-605-148), and IgG-Peroxidase (1:2000, Sigma-Aldrich, A6782,A0545

CHAPTER 3 RESULTS

3.1 Alternative first exon transcripts of the mouse *Nr3c1* gene

Work done as part of a publication:

"Differential regulation and function of 5'-Untranslated *GR*-exon 1 transcripts" Bockmühl Y, Murgatroyd C, **Kuczynska A,** Adcock M, Almeida OFX, Spengler D; *Molecular Endocrinology*, 2011

Contributions to the publication:

- involvement in revision process of the publication
- preparation of plasmid standards for quantification of GR 1st transcripts
- performing quantitative real-time PCRs
- involvement in writing the publication.

As described in the introduction, the promoter of the *Nr3c1* gene is composed of multiple 5'untranslated first exons, which give rise to various mRNA isoforms, but encode the same protein. While the structure of the rat and human *GR* 5'UTR were already established (Breslin *et al.* 2001; McCormick *et al.* 2000; Presul *et al.* 2007; Turner *et al.* 2005), the mouse GR promoter has been only partly characterised (F. Chen *et al*. 1999; Strähle *et al.* 1992). Therefore, in order to study epigenetic programming of the mouse *GR*, the structure of its 5'end had to be determined. Our major finding (Bockmühl *et al.* 2011) is the characterisation of five novel first exons (1.3, 1.4, 1.9 and 1.12), in addition to five previously described ones (1.1,1.5, 1.6, 1.10 and 1.11) (F. Chen *et al.* 1999; Strähle *et al.* 1992). A schematic summary of mouse *GR* promoters is presented in Figure 6.

3.1.1 Quantification and tissue distribution of *GR* first exons

We performed quantitative real-time PCR (qRT-PCR) in several central nervous system tissues (hippocampus, prefrontal cortex, hypothalamus), the pituitary, and various peripheral

tissues (liver, kidney, spleen, adrenals, and thymus), as well as in four clonal cell lines (corticotrope-derived AtT-20, hypothalamic N6, neuroblastoma N1E-115, and hippocampal progenitor HW3.5 cells). The absolute amounts for each exon were quantified based on a calibration curve, which was derived from amplification of set of standards of known concentration. Standards were obtained by serial dilutions of plasmid DNA, containing cloned partial target sequences. The forward primers were designed to be specific for each transcript, with the common reverse primer directed against exon 2. In addition, total *GR* was amplified using primers spanning coding exons 2 and 3. Expression of individual first exons was calculated as a percentage of total *GR* level in each tissue and cell line tested. Primer pairs used for expression analysis of mouse *GR* alternative first exons are listed in Table 1.

As shown in Figure 8, the overall expression patterns seem to be rather similar in all tested neuronal tissues and cell lines (with the exception of the most distal promoter 1.1), whereas peripheral tissues show more variations. Exon 1.1 is highly expressed in the brain and pituitary, as well as in spleen and thymus (~10-88%). Exons 1.6 and 1.11 are the most abundantly expressed (~10-50%); while exon 1.10 appears to be expressed at a moderate level (~5%) in all tested tissues and cell lines. In general transcripts 1.3, 1.7 and 1.9 display the lowest expression levels (~1% or less). However, 1.9 is slightly higher expressed in N1E-115, N6, and AtT-10 cell lines, and 1.3 in the hypothalamus and the hypothalamic N6 cell line, in comparison to other tissues tested.

Altogether, these results indicate that the expression of alternative *GR* first exons is not strictly tissue-specific. However, some alternative exons are preferentially expressed in certain tissues indicative of characteristic patterns of co-expressed transcript variants for each tissue and cell line tested, which contribute to total *GR* expression levels.

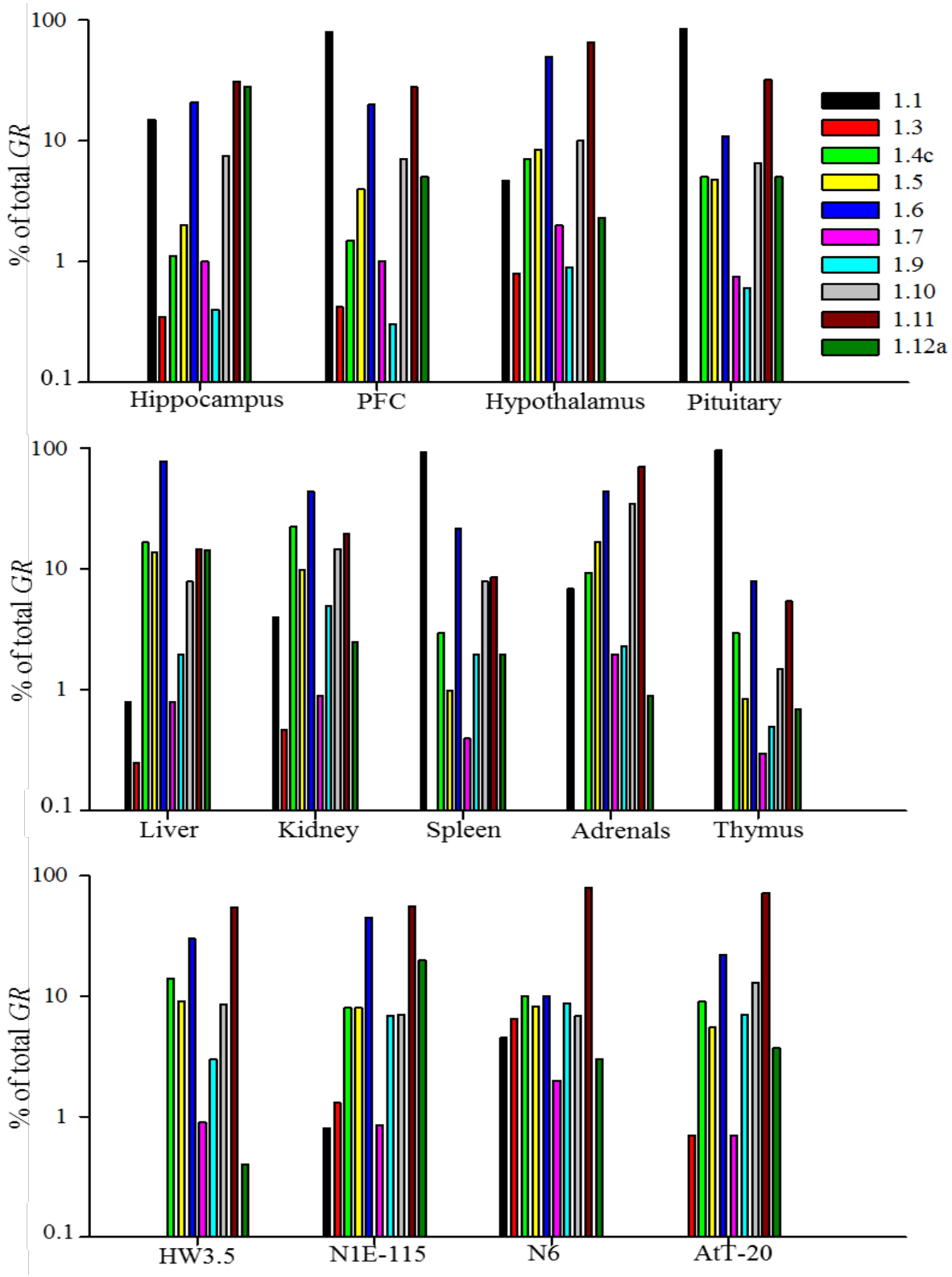

Figure 8: Expression and tissue distribution of *GR* first exon variants
Percentage contribution of alternative first exons of total *GR* mRNA shown in neuronal tissues: hippocampus, prefrontal cortex (PFC), and hypothalamus; peripheral tissues: pituitary, liver, kidney, spleen, adrenals, thymus, and cell lines: HW3.5, N1E-115, N6, and AtT-20. Copy numbers of each individual exon were calculated according to calibration curves and shown relative to the total copy number of total *GR* mRNA. Copy numbers were means evaluated for each transcript from two independent qPCR runs using independent preparations of DNA standards. Experiments were conducted jointly with Yvonne Bockmühl, post-doc.

3.1.2 Regulation of *GR* first exons expression

Beside tissue- and cell- type specific expression patterns, the alternative *GR* transcripts are thought to be regulated by various environmental signals (Yudt and Cidlowski 2002). Therefore, we assessed the expression of *GR* first exons in response to various cellular signalling pathways. Specifically, we treated AtT-20 and N6 cell lines with basic fibroblast growth factor (bFGF) or K^+-induced depolarization and subsequently analysed the expression of specific transcripts by quantitative RT-PCR.

Among other growth factor, bFGF is produced in the anterior pituitary, where it stimulates corticotropic cell proliferation (van Wijk *et al.* 1995). Our results show that transcription of individual promoters is differently regulated by a 24 h treatment of pituitary AtT-20 cells with bFGF. We observed a strong induction of 1.5 exon and to a lesser extend of 1.3, 1.4c, 1.6, 1.7, and 1.11 (Figure 9a). This up-regulation of specific *GR* exon first transcripts contributed to the overall increase in total *GR*.

It is well known, that neurons process and transmit their signals through cell membrane depolarization which can subsequently regulate the expression of certain genes (Flavell *et al*. 2008). Here, K^+ induced depolarization of hypothalamic N6 cells for 30 minutes induced the 1.11 (major transcript in N6 cells) and 1.4 transcripts, as well as slightly enhanced levels of total *GR*. Moreover, we observed decreased expression of the 1.9 transcript, without influencing the levels of the 1.3, 1.5, 1.6, 1.7, 1.10, and 1.12a transcripts (Figure 9b). Prolonged (2h) depolarisation further decreased the 1.9 transcript and down-regulated the one of 1.6. The expression of exon 1.1 was strongly induced, but as a minor transcript in N6 cells it did not alter total *GR*. Other transcripts were unchanged.

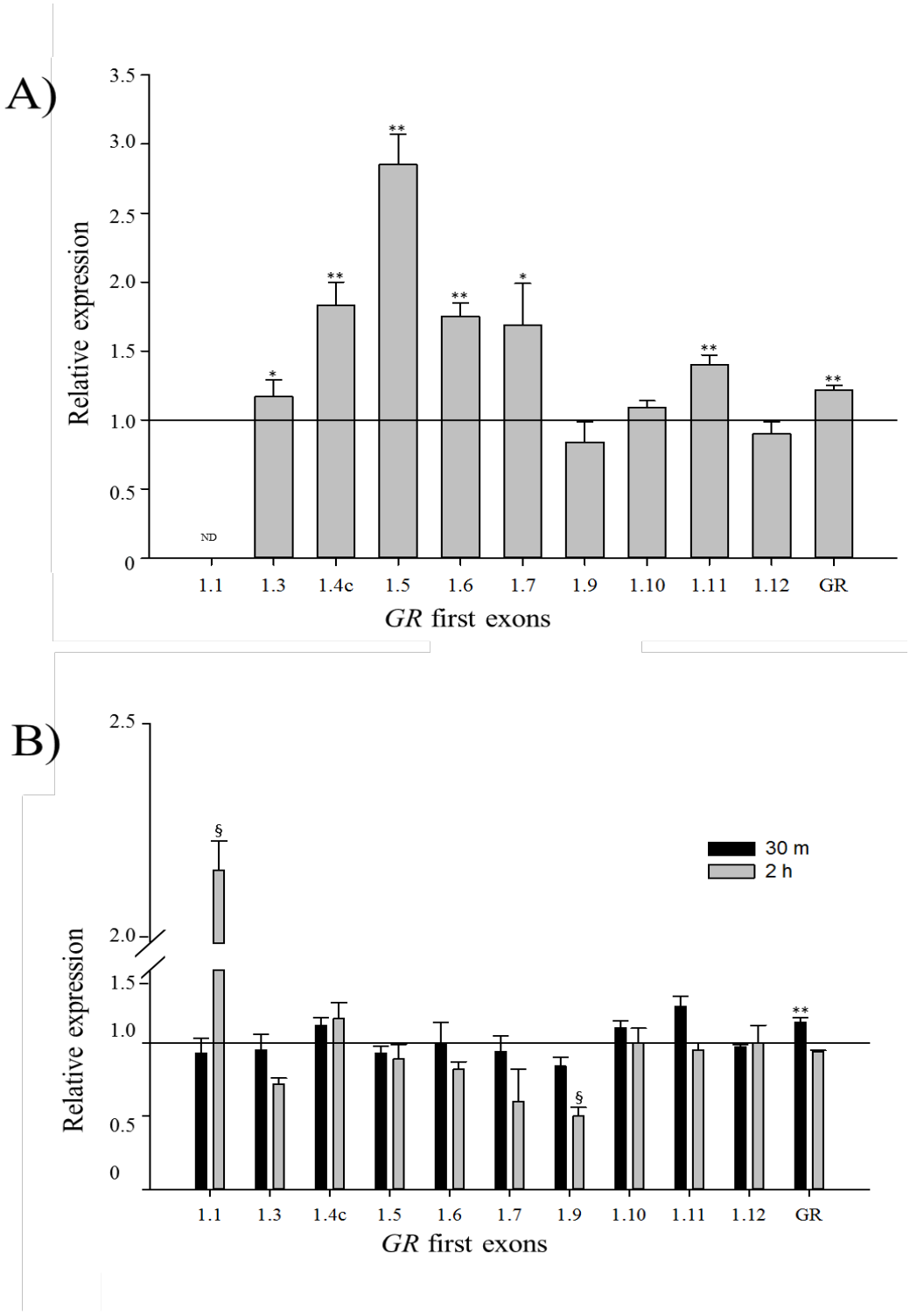

Figure 9: Regulation of *GR* first exon variants' expression by cellular signalling
A) Treatment of AtT-20 cell with the growth factor bFGF for 24h increases the expression of most of the identified transcripts, whereas others are not regulated. Expression of exon 1 transcripts was normalised to the internal standard Uros and Atp5j. Exon 1 transcripts of bFGF–treated cells are expressed as relative to expression of exon 1 transcripts in untreated cells. B) K^+-induced depolarization of N6 cells for 30 min and 2 h differentially regulates exon 1 transcripts. Expression of exon 1 transcripts was normalised to internal standard G6pdx. Exon 1 transcripts of depolarized cells are expressed as a function of expression of exon 1 transcripts in untreated cells. Data represent two independent experiments performed in triplicate (a) 2 or duplicate (b). Error bars are SEM (a) **P*< 0.04; ***P*< 0,003. (b) **P*< 0.025; § *P*<0.001, assessed by t-test. Experiments were done jointly with Yvonne Bockmühl, post-doc.

Moreover, it is important to note that, in addition to regulation of multiple *GR* first exons by cellular signalling (Figure 9), formation of alternative transcripts results in differential control of mRNA stability and translation efficiency as shown by (Bockmühl *et al.* 2011). This might add another layer for tight control of *GR* expression in a tissue- and cell- type specific manner.

3.2 Epigenetic programming of the *GR* by ELS

Work done as part of a publication:

"Epigenetic underpinnings of adverse experience-dependent resilience" Bockmühl Y, Patchev A, **Madejska A**, Hoffmann A, Sousa J, Sousa N, Holsboer F, Almeida OFX, Spengler D; *Epigenetics*, April 2015

Contributions to the publication:

- involvement in planning and performance of the experiments, including expression, methylation and chromatinimmunoprecypitation experiments

- micropunching of frozen brain tissue for methylation, expression and chromatinimmunoprecipitation experiments

- preparation of frozen brain tissue for IHC and ISH experiments

- involvement in writing the publication.

3.2.1 Conserved methylation pattern of ELS-responsive YY1 binding site

To exclude that our methylation results are strain specific and to show that changes in the degree of methylation of the ELS-responsive YY1 binding site can also potentially occur in another mouse strain than C57BL/6N inbred mice, we assessed the methylation pattern in CD1 outbred mice. Analyses were performed in 10 weeks old male mice without a history of ELS. Several brain areas (hippocampus, PFC, and hypothalamus) and peripheral blood mononuclear cells were compared to show that CpG island shore methylation is conserved in different tissues. Specifically, we analysed the methylation status of the region comprising CpG1-9. Hereby, CpG3 corresponds to the distal YY1 binding site (Figure 10).

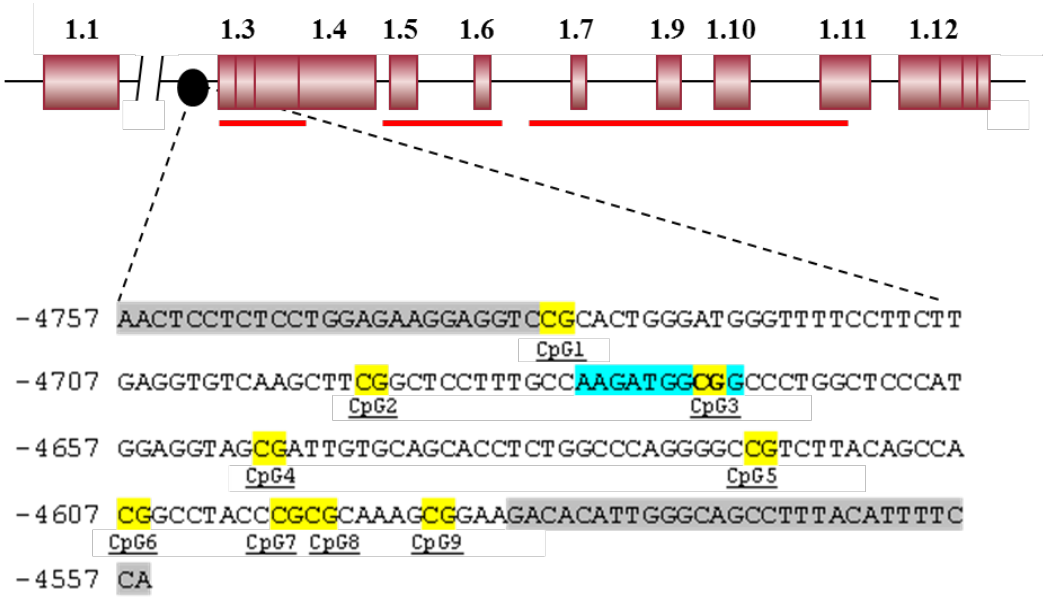

Figure 10: Sequence of mouse *GR* promoter fragment used for methylation analysis
The sequence at the CGI shore region of the mouse GR promoter, covering CpG1-9 (yellow), is shown. CpG3 corresponds to a putative YY1 transcription factor binding site (turquoise). Grey boxes represent sequences corresponding to bisulfite specific primers. Red lines correspond to CGIs.

Single clone bisulfite sequencing revealed similar methylation patterns of the CpG island shore region at the *GR* promoter between both mouse strains (Figure 11). In neuronal tissues of CD1 outbred mice we observed slightly higher DNA methylation at CpG3 (~25%) in comparison to C57BL/6N inbred mice (~10%). The methylation level in peripheral blood was comparable (20-25%) between both strains. These results confirmed that CpG methylation at the mouse YY1 binding site within the *GR* promoter is shared between inbred and outbred strains.

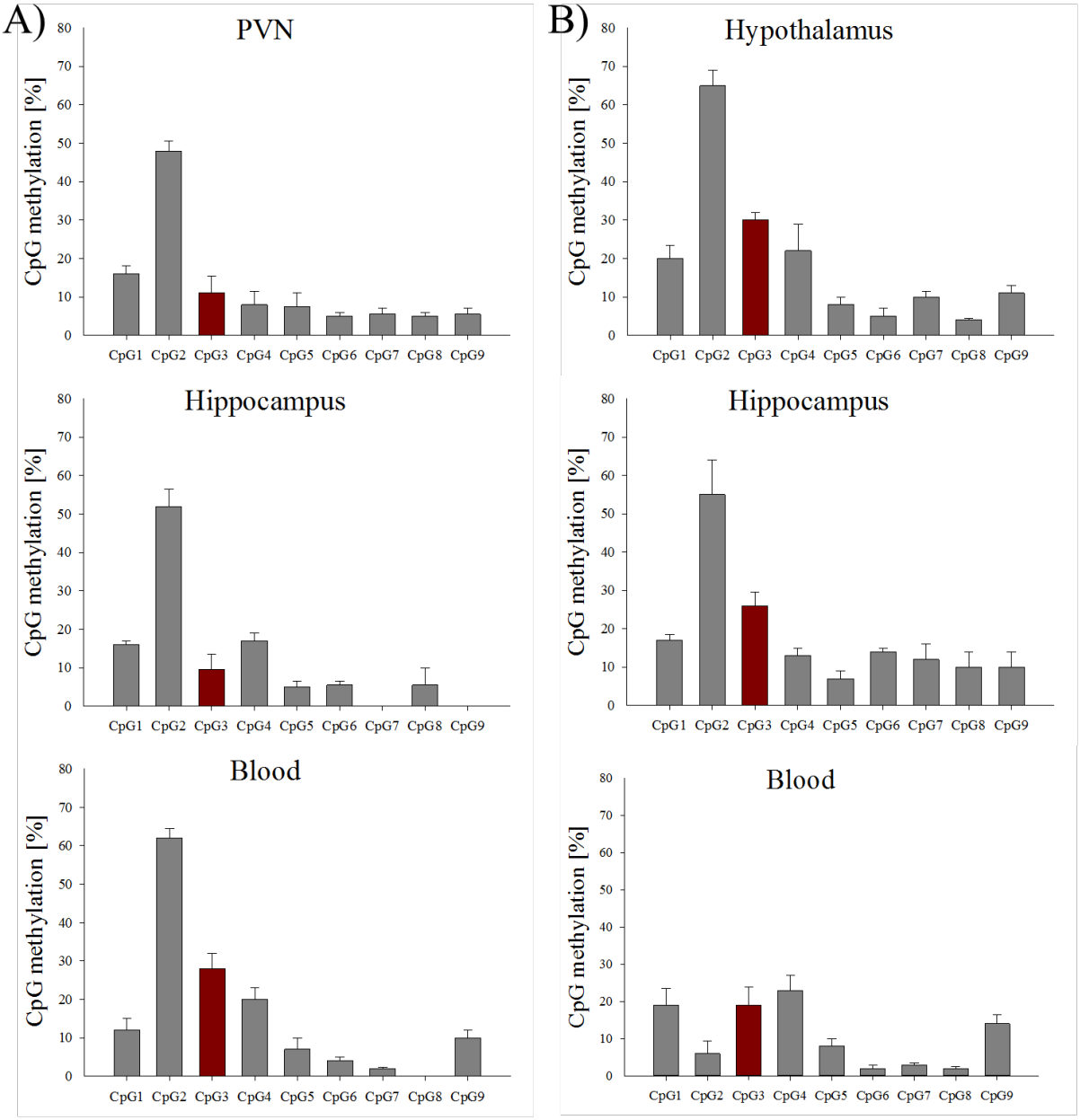

Figure 11: DNA methylation at the CGI shore region of the mouse *GR* promoter
CpG methylation was analysed by bisulfite sequencing and single clone reading using tissue from the PVN, hippocampus, and peripheral blood cells of 10 weeks old C57BL/6N inbred mice (n=5) A) and, hypothalamus, hippocampus, and peripheral blood cells of 10 weeks old CD1 outbred mice (n=5) B). 20-24 clones were sequenced per animal. The work was done jointly with Yvonne Bockmühl, post-doc.

Given the fact, that the structure of the human and mouse *GR* promoters is highly conserved, including the distal YY1 binding site [Figure 6, (Bockmühl *et al.* 2011; Breslin *et al.* 1998)], we asked next whether CpG methylation at these sites can be found also at the human *GR* promoter. Such findings could open the possibility of epigenetic programming of human *GR*

via mechanism related to YY1 binding. To address this topic, we initially performed sequencing analysis using bisulfite converted DNA isolated from various human neuroblastoma cell lines. In addition, we used peripheral blood mononuclear and buccal swap cells as well as post mortem brain samples (inferior cortex, hippocampus, and hypothalamus) obtained from male individuals without detectable brain pathology at the time of death.

We analysed the region of the human *GR* promoter sequence covering CpG1-11; human CpG7 is part of the putative distal YY1 binding site, identified previously (Breslin *et al.* 1998) and corresponds to mouse CpG3 (Figure12).

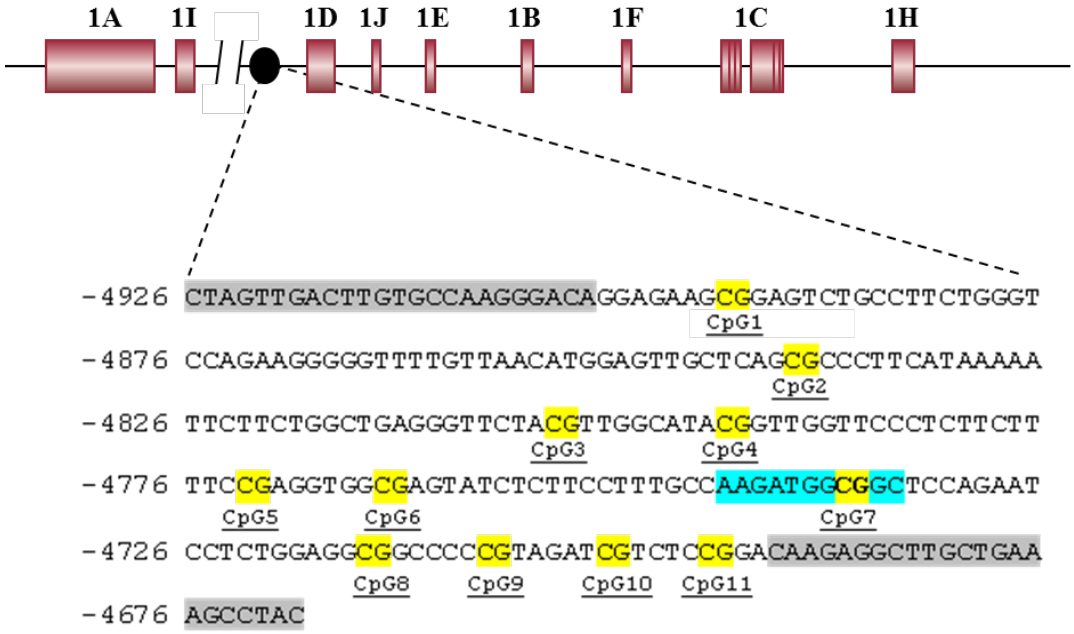

Figure 12: Sequence of the human *GR* promoter fragment used for methylation analysis
The sequence of the CGI shore region of the human *GR* promoter, covering analysed CpG1-11 (yellow). CpG7 corresponds to a putative distal YY1 transcription factor binding site (turquoise). Grey boxes represent sequences corresponding to bisulfite specific primers

Our studies revealed moderate methylation (5-10%) at CpG7 among all tested human cell lines as well as neuronal and peripheral tissues (Figure 13). Together with the mouse data, these results suggest cross-species conservation of the CGI shore region methylation pattern and a possible regulation by YY1 in a methylation-dependent manner. In view of these

findings, our post mortem studies open up the possibility for comparisons between the methylation patterns from control subjects and patients suffering from psychiatric disorders. Such findings could further strengthen the importance of YY1 binding for *GR* expression. However, due to limited access to post mortem brain tissues from control subjects and patients, we were unable to answer this question.

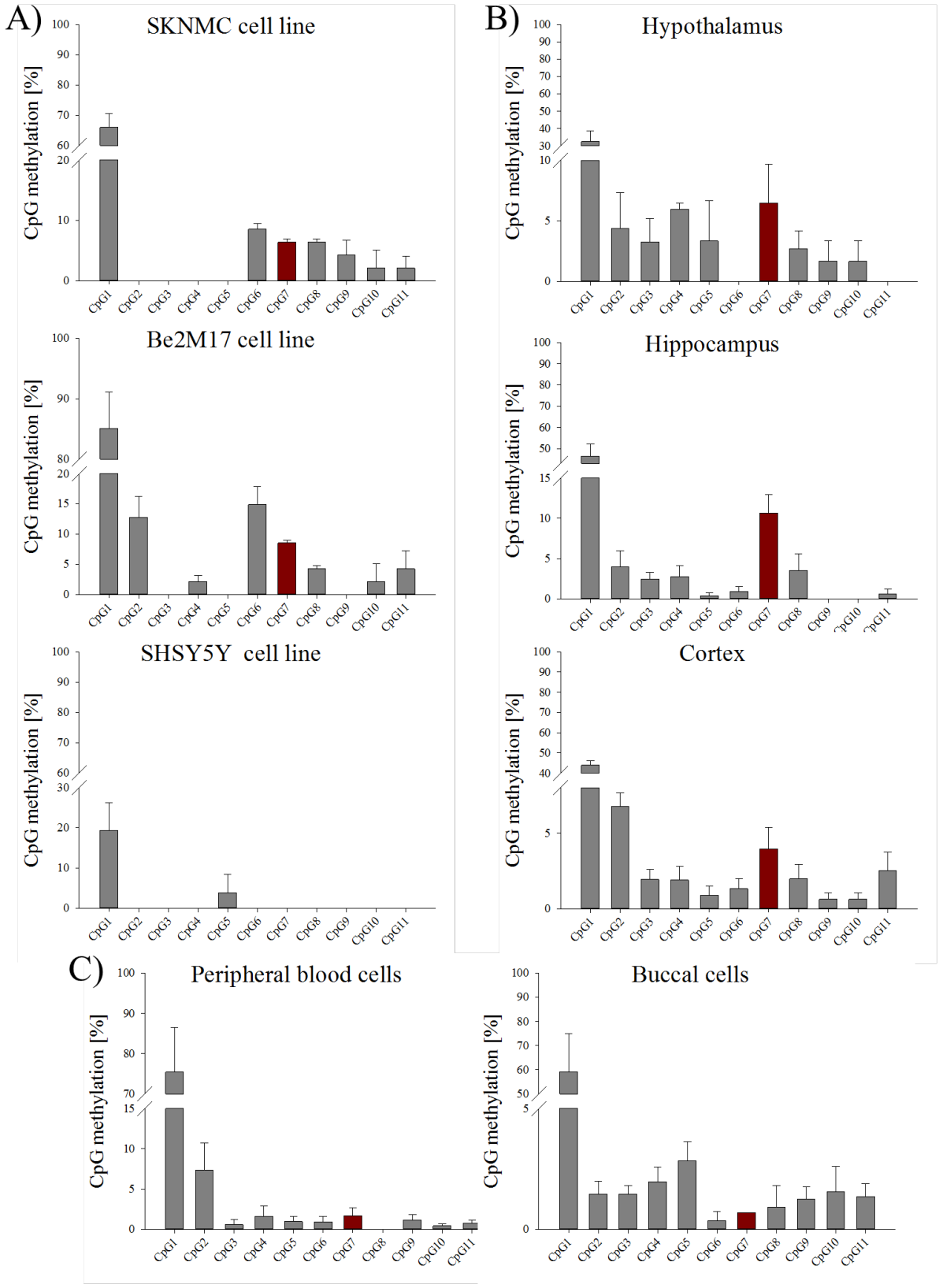

Figure 13: DNA methylation at the CGI shore region of the human *GR* promoter
CpG methylation was analysed by bisulfite sequencing and single clone reading in the human neuroblastoma cell lines (SKNMC, Be2M17, and SHSY5Y) and A) post mortem brain samples (hypothalamus n=4, hippocampus n=6, and cortex n=10) obtained from control subjects and, B) buccal swap and peripheral blood cells from healthy donors n=11 C). 20-24 clones were sequenced per sample.

3.2.2 Up-regulation of multiple *GR* first exons in ELS mice

As shown previously, the *GR* is composed of multiple 5'-untranslated first exons, which give rise to the same protein (Bockmühl *et al.* 2011; Breslin *et al.* 2001; Turner *et al.* 2005). Moreover, some of the identified *GR* first exons might be differentially regulated by various stimuli, including growth factor- and depolarization-dependent signalling (Figure 9). In order to investigate if specific *GR* transcripts are regulated by ELS, leading to changes of total *GR* mRNA, quantitative RT-PCR was performed with RNA isolated from PVNs of 10 days and 3 month old control and ELS mice. This experiment revealed strong up-regulation of 1.4c and 1.7 transcripts in 10 days old mice with a history of maternal separation (Figure 14a). However, as less prevalent transcripts, they contribute weakly to total *GR*, the expression of which is not changed in these mice (Bockmühl *et al.* submitted). On the other hand, in 3 month old ELS mice we observed an up-regulation of multiple *GR* transcripts; including major 1.6 and 1.11, which contribute strongly to increased total *GR* mRNA expression. Strong induction of minor transcripts 1.9 and 1.12 had probably less effect on the final result. Noticeably, 1.7 exon was not altered (Figure 14b).

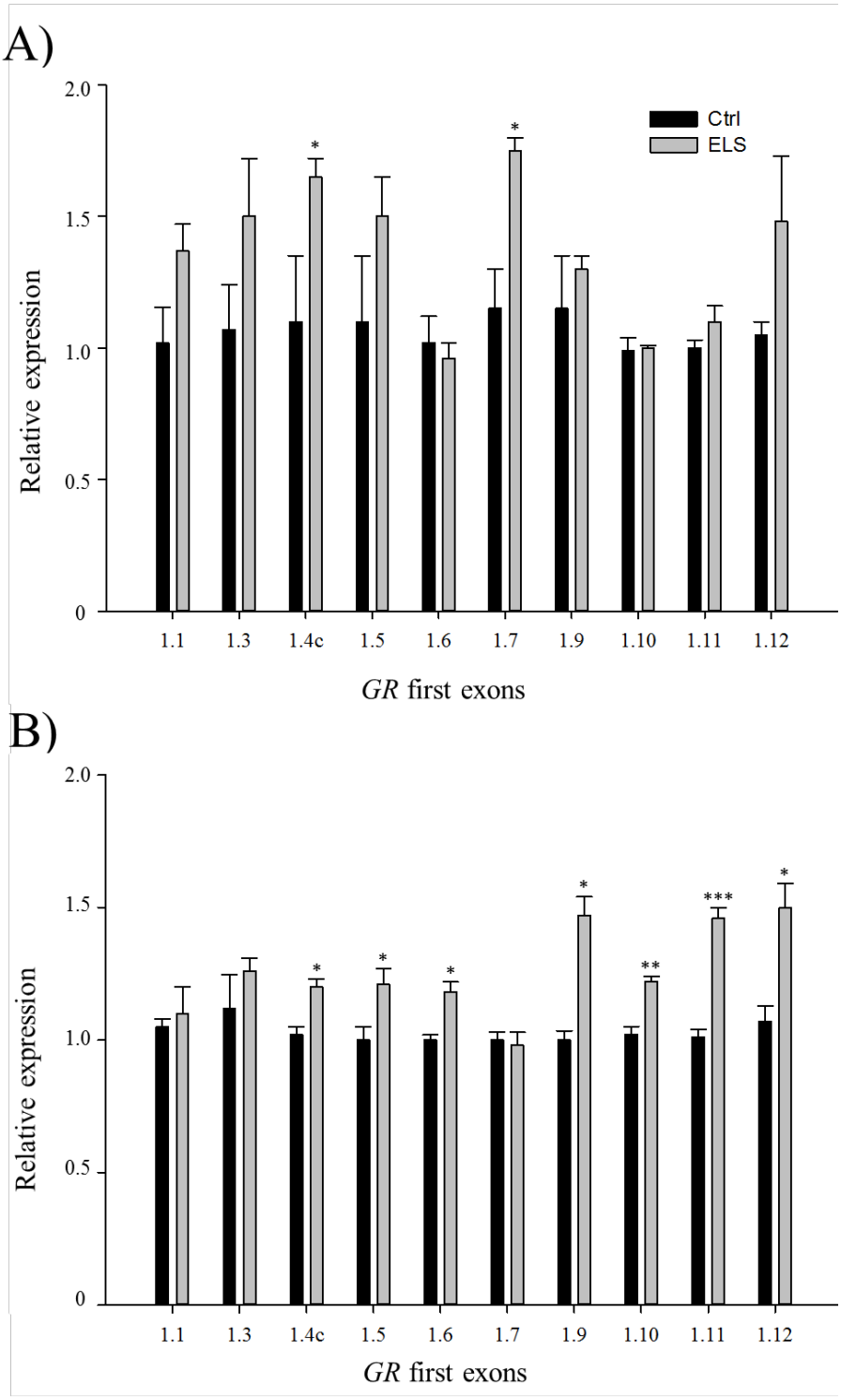

Figure 14: Regulation of *GR* first exons in PVN by ELS

GR exon 1 transcripts were quantified by quantitative RT-PCR in 10 days A) and 3 month B) old ELS and Ctrl mice (n = 7-9 per group). Data are shown relative to the expression levels in the control group and normalized with *Atpj5* as internal reference and relative to the expression of the Ctrl group (n = 7-9). Error bars are SEM *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$, assessed by t-test. These experiments were done jointly with Yvonne Bockmühl, post-doc.

3.2.3 GR protein levels in the PVN

GR protein expression in the PVN was assessed by immunohistochemistry (IHC). Triple staining of GR, Crh and Avp was performed on PVN sections obtained from mice with a history of MS or controls. Our findings of higher GR protein levels in ELS mice (Figure 15) argue against an impaired negative feedback. IHC revealed unaltered Crh expression in ELS mice and Avp up-regulation, as shown previously (Murgatroyd *et al.* 2009). Interestingly, as evidenced in Figure 15b, GR expression is restricted to Crh positive neurons and was absent in Avp positive neurons indication that GR up-regulation in response to ELS occurred in a cell-type specific manner.

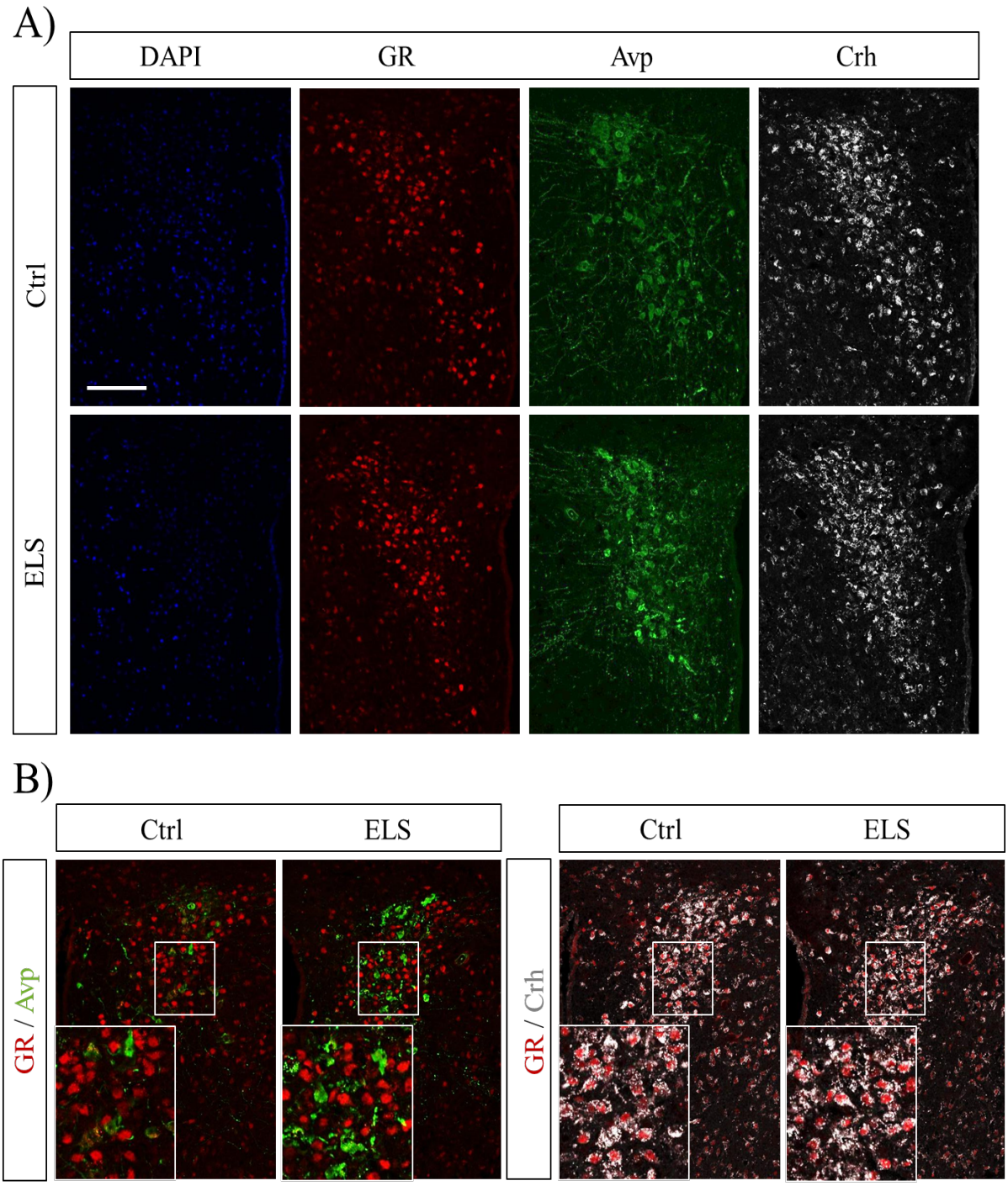

Figure 15: Representative images of immunohistochemical triple-staining to detect GR, Crh and Avp

ELS led to increased immunostaining of GR and Avp, having no influence on Crh A). Colocalization of GR/Crh (right panel) and lack of colocalization GR/Avp (left panel) on PVN sections from control Ctrl and ELS mice B). Scale bar = 50 µm. Primary and secondary antibodies used for triple-staining of GR, Avp and Crh are listed in Table 5. Work done jointly with Yvonne Bockmühl, post-doc.

3.2.4 GR signalling in ELS mice

Given the fact that GR serves as a main regulator of the HPA axis, its proper expression and function is necessary for sufficient negative feedback regulation, what is especially important in the context of different pathologies (de Kloet *et al.* 2005). In this respect, impaired GR signalling has been postulated as a key mechanism involved in the hyperactivity of the HPA axis (Anacker *et al.* 2011; Holsboer 2000). Previous work in our laboratory revealed that maternal separation, used to induce ELS in mice, leads to sustained hyperactivity of the HPA axis. For that reason, we asked whether elevated GR protein levels in ELS mice resulted in enhanced transcriptional activity. To address this question, we investigated the expression profiles of classical target genes. Several candidates were selected that were shown earlier to be inducible in rodent hypothalamus by corticosterone or stress, such as genes coding for: FK506 binding protein 5 (*Fkbp5*) (Hubler *et al.* 2004); serum-and glucocorticoid-induced protein kinase (*Sgk1*) (Sarabdjitsingh *et al.* 2010); Glucocorticoid-induced leucine zipper (*Gilz*) (Sarabdjitsingh *et al.* 2010); down-regulated in renal cell carcinoma 1 (*Drr1*) (Schmidt *et al.* 2011); pyruvate dehydrogenase kinase 4 (*Pdk4*) (Ding *et al.* 2010); and dual specificity phosphatase 1 (*Dusp1*) (Tchen *et al.* 2010). We performed qRT-PCR using primers listed in Table 1. Analysis was performed in PVNs of 10 weeks old male mice (control / ELS) that have received a single acute intraperitoneal (ip) injection of corticosterone (0.1; 1.0 and 10.0 mg/kg). Animals were sacrificed 3 hours after injection. Tissue from the dentate gyrus, where *GR* mRNA levels did not differ between control and ELS mice, was used as a negative control In concordance with previously published data (Scharf *et al.* 2011), corticosterone treatment resulted in a dose-dependent increased expression of all tested GR target genes in PVN (Figure 1b). As we did not observe differential expression of these genes after intraperitoneal saline injection, the data from corticosterone application were referred to those from saline application which were set to one. The data were analysed by two- way ANOVA and show

that maternal separation (stress) enhanced GR protein levels in the PVN and significantly increased the expression of the *Fkbp5*, *Sgk1* and *Dusp1* genes ($p<0.001$, $p< 0.1$, $p<0.1$), respectively (Table 6). These findings can be explained by the fact that *Fkbp5*, *Sgk1* and *Dusp1*encode a maximum of two GREs (Arteaga *et al.* 2008; Hubler *et al.* 2004; Tchen *et al.* 2010). Therfore, their inducibility may be facilitated when GR expression is increased. On the other hand, *Gilz, Drr1*, and *Pdk4* do not show an effect of stress. Nevertheless, they all show an significant effect of CORT injection ($p<0.0001$, $p< 0.0001$, $p< 0.000001$ respectively) similar to *Fkbp5*, *Sgk1* and *Dusp1* ($p<0.0001$, $p< 0.001$, $p<0.0001$). This shows that all of these genes are responsive to CORT, but only some are directly regulated (their expression is influenced by ELS-induced GR up-regulation). There is no interaction effect of stress x CORT observed for all tested genes. Altogether, our expression data suggest that the level of activated GR is important in the regulation of certain target genes while others behaving largely indistinguishably between ELS and control mice might be regulated by additional mechanisms unrelated to GR.

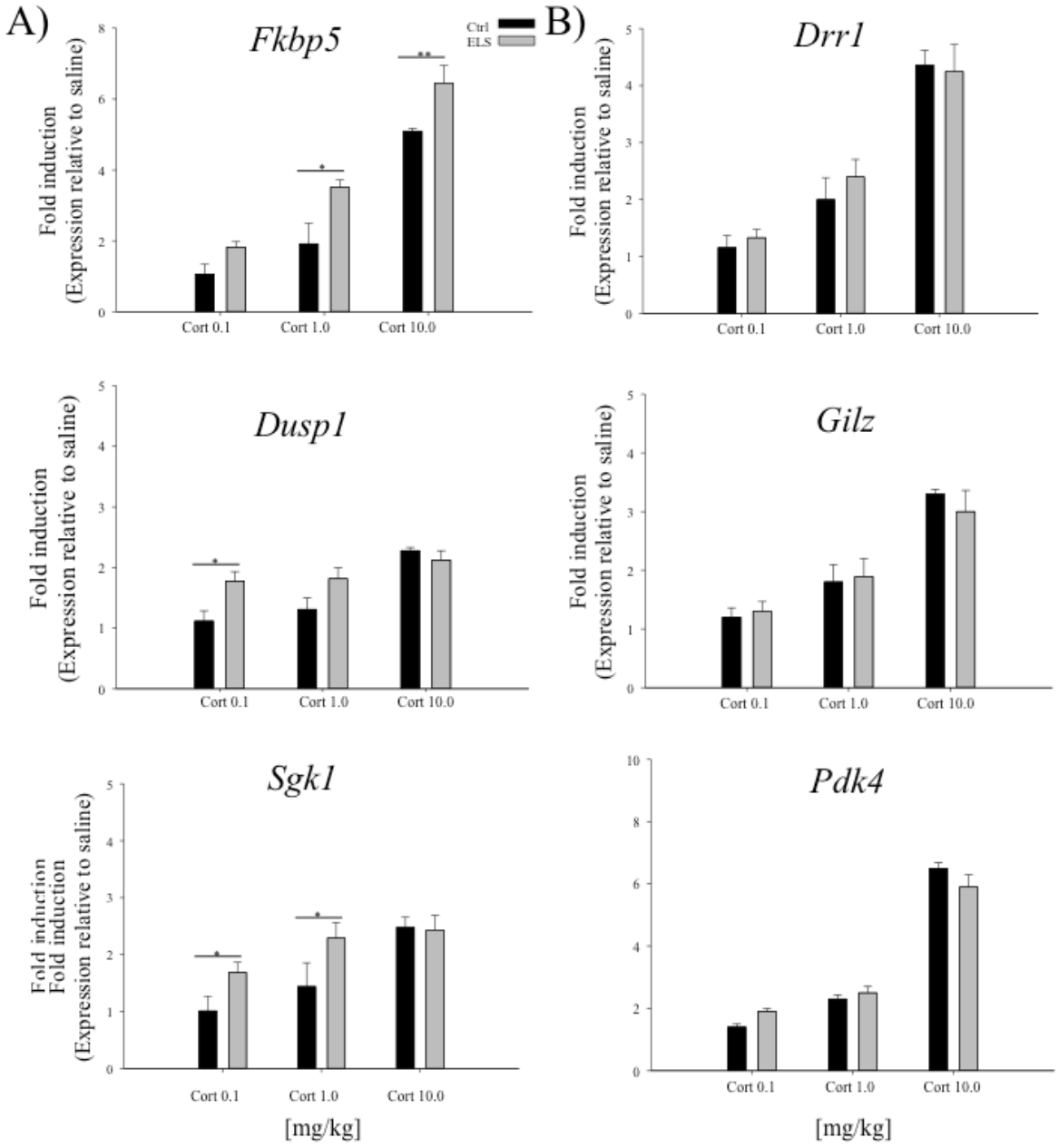

Figure 16: Expression of GR target genes in the hypothalamic PVN
Intraperitoneally injected corticosterone (Cort; 0.1, 1.0, 10.0 mg/kg) induced GR-responsive genes in the PVN of 10-weeks old Ctrl and ELS mice, as measured by qPCR. Increased GR expression in ELS mice translates to higher GR transactivation, as shown by higher expression of some GR target genes, containing in their sequence appropriate GREs. Expression data are normalized to *Atpj5*; fold-induction is shown; Error bars are SEM.; n = 4-8 animal per group. Data were analysed by two -way ANOVA (Stress, CORT) with Stress (ELS,Ctrl) and CORT (0.1, 1.0, 10 mg/kg), followed by Newman-Keuls post-hoc test, if appropriate (statistical analysis are shown in Table 6); * $p < 0.05$, ** $p < 0.01$

Protein	Stress	CORT	Stress x CORT
Fkbp5	$F_{(1,30)} = 19.85$, ***p*< 0.001**	$F_{(2,30)} = 63.65$, ***p*< 0.0001**	$F_{(2,30)} = 1.644$, $p = 0.210$
Sgk1	$F_{(1,31)} = 4.27$, ***p*< 0.1**	$F_{(2,31)} = 6.97$, ***p*< 0.001**	$F_{(2,31)} = 1.30$, $p = 0.28$
Drr1	$F_{(1,29)} = 0.29$, $p = 0.59$	$F_{(2,29)} = 38.52$, ***p*< 0.0001**	$F_{(2,29)} = 0.27$, $p = 0.76$
Gilz	$F_{(1,31)} = 0.04$, $p = 0.83$	$F_{(2,31)} = 21.20$, ***p*< 0.0001**	$F_{(2,31)} = 0.75$, $p = 0.47$
Dusp1	$F_{(1,27)} = 6.01$, ***p*< 0.1**	$F_{(2,27)} = 11.05$, ***p*< 0.01**	$F_{(2,27)} = 3.12$, $p = 0.06$
Pdk4	$F_{(1,31)} = 0.00055$, $p = 0.98$	$F_{(2,31)} = 28.42$, ***p*< 0.000001**	$F_{(2,31)} = 1.05$, $p = 0.36$

Table 6: Statistical analysis of Fig. 16

In the next step we analysed the expression of the same set of GR target genes in the dentate gyrus (DG). As shown in our previous work, the DG is a tissue for which we did not observe differential expression of *GR* in response to maternal separation (Bockmühl *et al.* submitted). We analysed the expression of *Fkbp5, Sgk1* (in the PVN their expression is induced stronger in ELS mice following CORT injection) and *Drr1* (in the PVN no differences between naïve and ELS mice following CORT injection). The data were analysed by two- way ANOVA and, in the case of *Fkbp5* and *Sgk1*, we did not observe any effect of stress. *Fkbp5* shows a significant effect of CORT injection ($p<0.0001$ (Table 7)). Similar to previously reported results (Scharf *et al.* 2011), we observed that in tissues with low basal expression of *Fkbp5* (e.g.; PVN) induction of its expression is much stronger than in tissues with high basal expression (e.g.; hippocampus) (Figure 16 and 17). Moreover, in hippocampal DG single corticosterone injection did not affect *Sgk1* mRNA expression, what is in concordance with another earlier study (van Gemert *et al.* 2006). On the other hand, *Drr1* gene shows a stress effect ($p< 0.001$ (Table 7)), suggesting additional layers of gene regulation, independent of GR signaling. There is no interaction effect of stress x CORT for all tested genes.

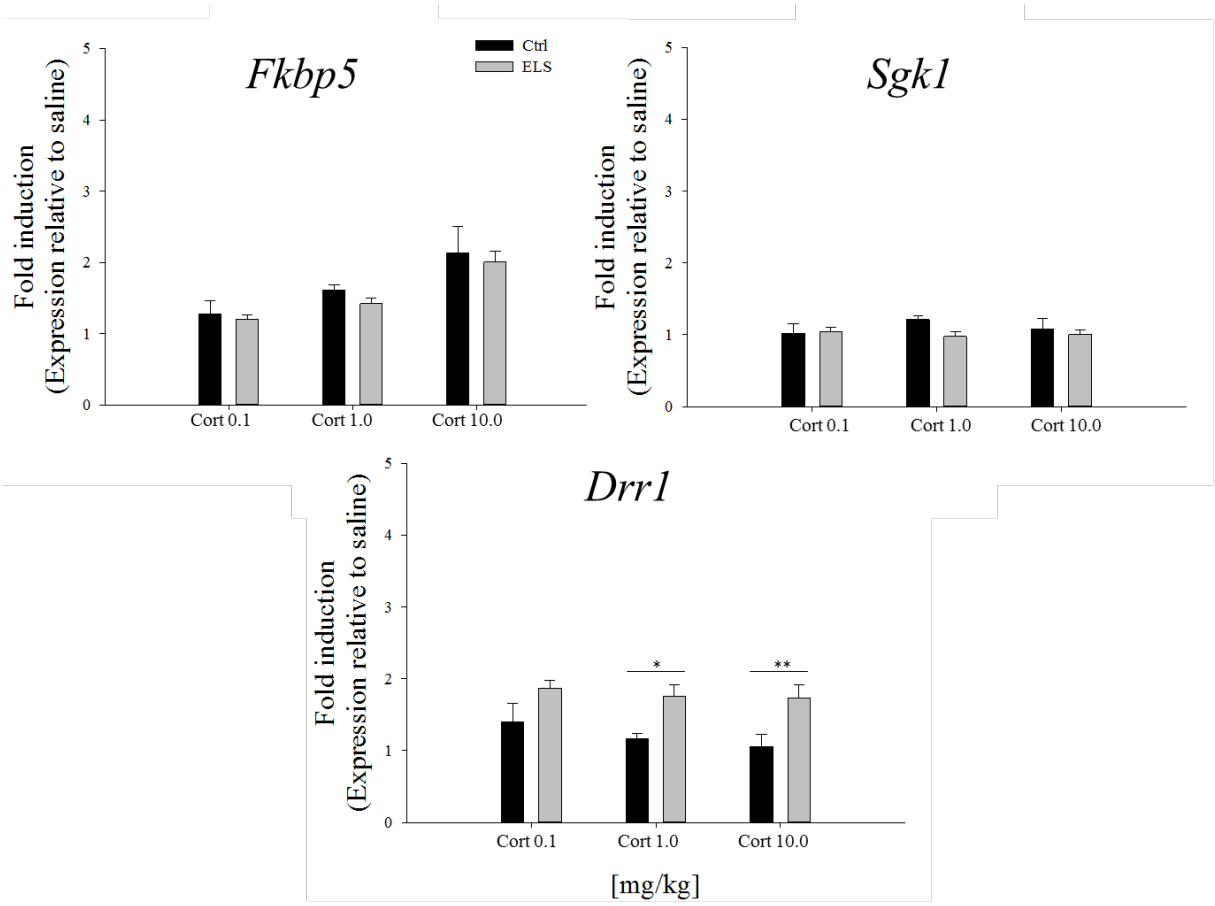

Figure 17: Expression of GR target genes in the hippocampal DG
Intraperitoneally injected corticosterone (Cort; 0.1, 1.0, 10.0 mg/kg) induced slightly *Fkbp5*, and did not change *Sgk1* and *Drr1* expression in the DG of Ctrl and ELS mice, as measured by qPCR. In contrast to the PVN, *Fkbp5* and *Sgk1* expression did not differ between Ctrl and ELS mice; increased *Drr1*expression in ELS mice was observed with higher Cort doses. Expression data are normalized to *Atpj5*; fold-induction is shown; Error bars are SEM; n = 4-8 animal per group. Data were analysed by two -way ANOVA (Stress, CORT) with Stress (ELS,Ctrl) and CORT (0.1, 1.0, 10 mg/kg), followed by Newman-Keuls post-hoc test, if appropriate (analysis shown in Table 7); * $p< 0.05$, ** $p< 0.01$

Protein	Stress	CORT	Stress x CORT
Fkbp5	$F_{(1,34)} = 1.08$, $p=0.30$	$F_{(2,34)} = 13.77$, $\mathbf{p< 0.0001}$	$F_{(2,34)} = 0.32$, $p< 0.72$
Sgk1	$F_{(1,34)} = 2.17$, $p=0.14$	$F_{(2,34)} = 0.34$, $p= 0.7$	$F_{(2,34)} = 1.230$, $p= 0.3$
Drr1	$F_{(1,34)} = 19.25$, $\mathbf{p< 0.001}$	$F_{(2,34)} = 1.13$, $p= 0.33$	$F_{(2,34)} = 0.19$, $p= 0.82$

Table 7: Statistical analysis of Fig. 17

3.2.5 Expression analysis of genes down-regulated by GR in PVN

In addition, we assessed the expression of crucial components of the HPA axis known to be repressed by GR, i.e.; *Avp* and *Crh*. A two- way ANOVA analysis revealed neither significant effects of stress nor CORT injection. There is also no significant stress x CORT interaction observed. In opposite to our expectations, we were unable to detect their down-regulation with increasing doses corticosterone by qRT-PCR (Figure 18). Similarly, *in situ* hybridisation carried out on PVN sections obtained from mice injected with 1.0 mg/kg CORT, using a radioactively labelled probe against *Avp* and *Crh* did not show decreased mRNA expression (data not shown). It is important to mention, that our IHC results (Figure 15b) revealed no co-localization between GR and Avp.

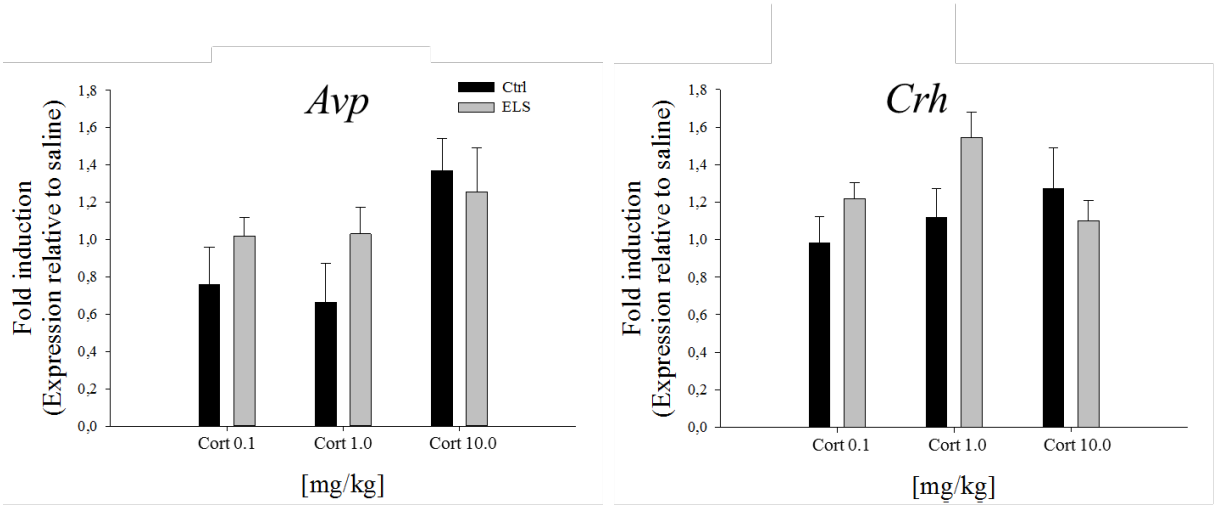

Figure 18: Expression of genes down-regulated by GR
Intraperitoneally injected corticosterone (Cort; 0.1, 1.0, 10.0 mg/kg) did not regulate significantly *Avp* and *Crh* expression in the PVN, as measured by qPCR. Expression data are normalized to *Atpj5*; fold-induction is shown; Error bars are SEM; n = 4-8 animal per group. Data were analysed by two- way ANOVA (Stress, CORT) with Stress (ELS,Ctrl) and CORT (0.1, 1.0, 10 mg/kg). Analysis shown in Table 8

Protein	Stress	CORT	Stress x CORT
Avp	$F_{(1,31)} = 1.09$, $p = 0.30$	$F_{(2,31)} = 3.19$, $p = 0.54$	$F_{(2,31)} = 0.77$, $p = 0.47$
Crh	$F_{(1,31)} = 2.79$, $p = 0.1$	$F_{(2,31)} = 1.16$, $p = 0.32$	$F_{(2,31)} = 2.42$, $p = 0.1$

Table 8: Statistical analysis of Fig. 18

3.2.6 GR occupancy at exemplary GREs in the PVN

Following activation by GCs, glucocorticoid receptors translocate to the nucleus and exert their effects by binding to specific glucocorticoid response elements (GREs) located within regulatory regions of GR target genes (van der Laan *et al.* 2008). Since our expression analysis (Figure 16a) have shown differential regulation of three GR target genes (*Fkbp5*, *Sgk1*, and *Dusp1*), we performed an *in vivo* chromatin immunoprecipitation (ChIP) assay using PVNs of 10 weeks old male mice (control/ELS) injected intraperitoneally with one dose of corticosterone (1.0 mg/kg) or saline. Animals were sacrificed 90 minutes after injection. The recovered DNA containing target gene sequences for GR from the PVNs was clearly enriched after immunoprecipitation with the anti-GR antibody, as compared to the negative control without antibody (data not shown). The data presented here (Figure 19) were analysed by two- way ANOVA (Table 9) and support the hypothesis that, in comparison to controls, elevated GR levels in ELS mice lead to higher occupancy at the intronic DNA-binding site of *Fkbp5* and one of the two sites of *Dusp1* (GRR-29). Therefore, this set of data proves the functionality and increased signalling of up-regulated glucocorticoid receptors in ELS mice in the PVN. In case of *Sgk1* we did not detect any significant differences in GR occupancy at the GRE located distally from the TSS between animals with a history of maternal separation and controls.

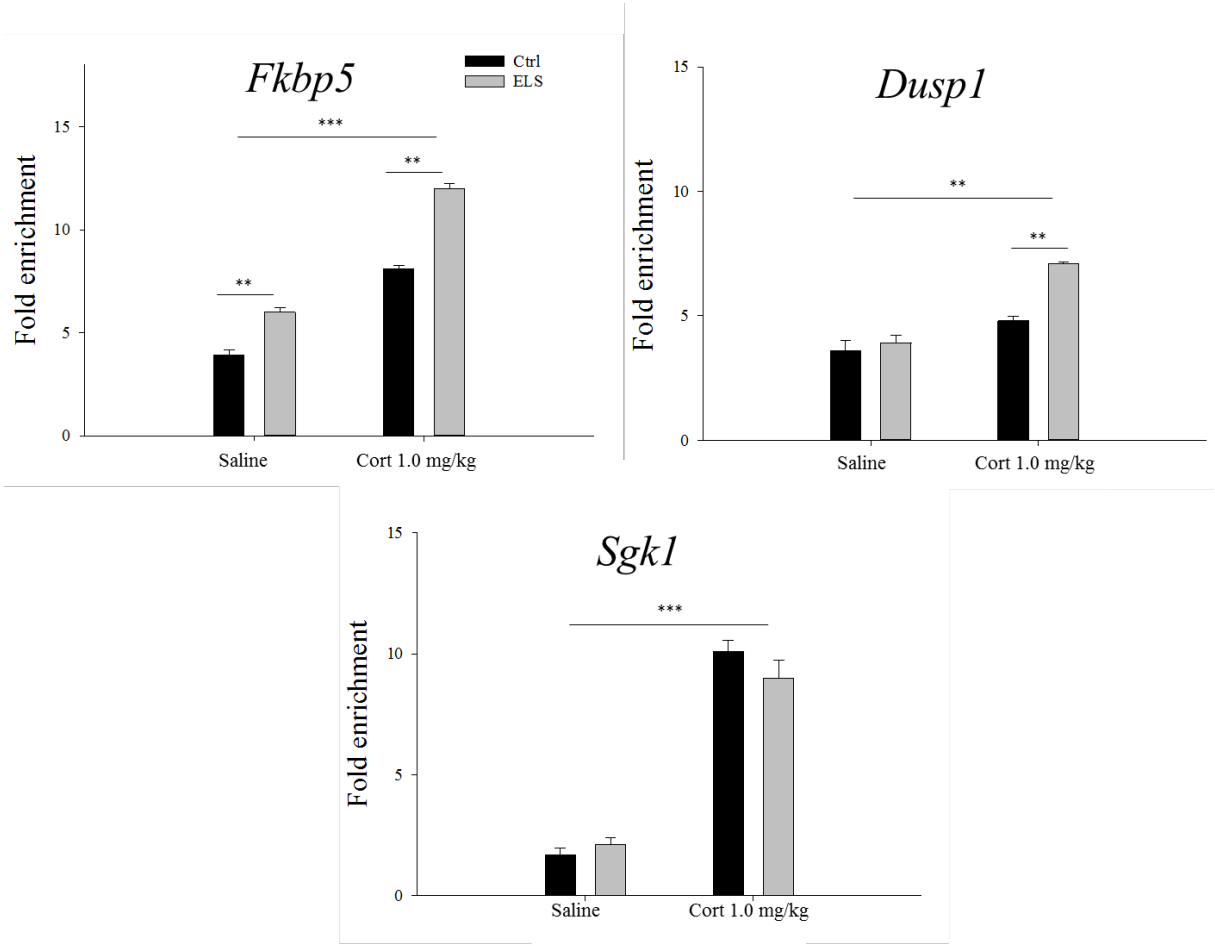

Figure 19: GR occupancy at GREs of exemplary target genes in the PVN
Increased GR expression in ELS mice translates to higher GR occupancy at regulatory regions of exemplary target genes, as shown by in vivo ChIP. Recovered DNA was analysed by qPCR and normalized to non-precipitated input chromatin. For this, 3 PVNs from 10-week old mice were pooled for 5 ChIP analysis (n = 15 per group). Error bars are SEM. *P< 0.05. Data were analysed by two- way ANOVA (Stress, CORT) with Stress (ELS,Ctrl) and CORT (1.0 mg/kg), followed by Newman-Keuls post-hoc test, if appropriate (analysis shown in Table 9) *** p< 0.001, ** p< 0.01, * p< 0.05

Protein	Stress	CORT	Stress x CORT
Fkbp5	$F_{(1,14)} = 6.58$, ***p*< 0.1**	$F_{(2,14)} = 17.49$, ***p*< 0.001**	$F_{(2,14)} = 0.26$, $p = 0.61$
Sgk1	$F_{(1,14)} = 0.34$, $p = 0.56$	$F_{(2,14)} = 25.30$, ***p*< 0.001**	$F_{(2,14)} = 1.66$, $p = 0.21$
Dusp1	$F_{(1,14)} = 4.28$, ***p*< 0.1**	$F_{(2,14)} = 14.54$, ***p*< 0.001**	$F_{(2,14)} = 1.17$, $p = 0.35$

Table 9: Statistical analysis of Fig. 19

3.2.7 GR occupancy at exemplary GREs in Dentate Gyrus

To strengthen our hypothesis of important ELS-dependent differences in *GR* expression in the PVN, we investigated the GR occupancy at GREs in the dentate gyrus as well. As expected,

no significant differences in GR binding to GREs of exemplary target genes were reported (Figure 20).

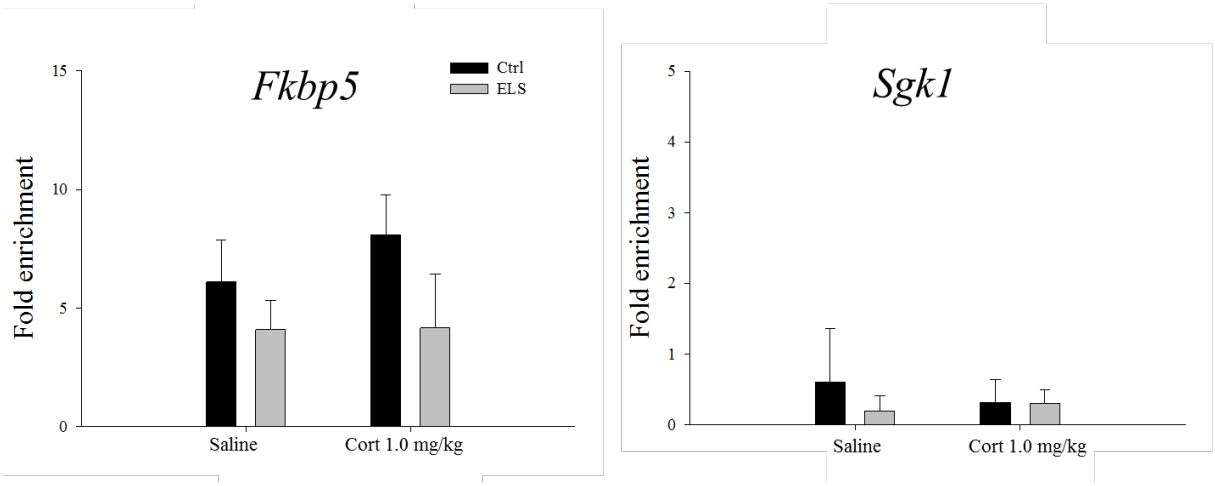

Figure 20: GR occupancy at GREs of exemplary target genes in the DG
ELS did not increase GR occupancy at regulatory regions of exemplary target genes, as shown by *in vivo* ChIP. Recovered DNA was analysed by qPCR and normalized to non-precipitated input chromatin. For this, DG from three 10-week old mice were pooled for 5 ChIP experiments (n = 15 per group). Error bars are SEM. Data were analysed by two- way ANOVA (Stress, CORT) with Stress (ELS,Ctrl) and CORT (1.0 mg/kg),

Protein	Stress	CORT	Stress x CORT
Fkbp5	$F_{(1,16)} = 1.90$, $p = 0.18$	$F_{(2,16)} = 0.32$, $p = 0.58$	$F_{(2,16)} = 0.048$, $p = 0.82$
Sgk1	$F_{(1,16)} = 1.07$, $p = 0.82$	$F_{(2,16)} = 1.03$, $p = 0.46$	$F_{(2,16)} = 0.28$, $p = 0.57$

Table 10: Statistical analysis of Fig. 20

CHAPTER 4 DISCUSSION

In the present work we focused on epigenetic programming of *GR* by early-life stress. For that purpose, in the first part of the study, it was necessary to establish the structure of the mouse *GR* promoter, as well as tissue distribution of *GR* alternative first exons and their regulation by various signaling pathways. We found that, although most of alternative first exons are widely expressed in different brain and peripheral tissues and in clonal cell lines, their transcription can be differently regulated by growth factor- and depolarization-induced signaling.

As already stated in introduction, *GR* gene expression is highly dependent on multiple untranslated first exons, which give rise to alternate RNA isoforms, but encode the same protein. While the composition of the rat and human *GR* promoters was known (Breslin *et al.* 2001; Turner *et al.* 2005; Presul *et al.* 2007; McCormick *et al.* 2000), knowledge of the mouse homolog was missing. Therefore, we carried out a detailed analysis and identified four new transcripts in addition to five previously described untranslated first exons (Strähle *et al.* 1992). These findings have been recently published (Bockmühl *et al.* 2011).

Moreover, the tissue-specific distribution of different first exons has been examined previously in a wide range of human and rat tissue (Alt *et al.* 2010; McCormick *et al.* 2000; Turner *et al.* 2005) and to a lesser extent in mouse (Strähle *et al.* 1992). For that reason absolute quantification and tissue distribution of *GR* first exons were analysed. Interestingly, we did not detect clear tissue-specific expression patterns of mouse *GR* alternative first exons (Figure 8). However, as shown for human (Turner *et al.* 2005; Alt *et al.* 2010), mouse *GR* transcripts display a conserved expression pattern across all neuronal tissues tested, whereas more variations were reported throughout peripheral tissues. We observed also some between-species discrepancies. Whereas, human 1B and 1C (Alt *et al.* 2010; Breslin *et al.* 2001; Turner *et al.* 2005), and their rat homologs 1.6 and 1.10 (McCormick *et al.* 2000) are reported to be

the most abundantly expressed, we showed 1.6 and 1.11 (but not 1.10) to be the major transcripts among all tested mouse tissues. Moreover, the most distal human 1A (Presul *et al.* 2007), rat 1.1 (McCormick *et al.* 2000) and mouse 1.1 (previously known as 1A) (Strähle *et al.* 1992) were reported to be specific for hemapoetic cell lines and thymocytes. In the present study, we detected 1.1 to be abundantly expressed also in brain (hippocampus, PFC, and hypothalamus), pituitary, thymus, adrenals, and spleen, and to lesser extend in kidney and liver. Importantly, as most of the researches investigate currently the expression of rat 1.7 transcript (Weaver *et al.* 2004) and its human homolog 1F (McGowan *et al.* 2009), we show that mouse 1.7 is a minor transcript in all tested tissues and represents ~1 % of total *GR* mRNA. Similar levels of 1F expression (~1% of total *GR* mRNA) in human were detected by Alt *et al.* (2010). These results are in contrast to those of McGowan *et al.* (2009) who reported this transcript to contribute by 40-60% to the total amount of *GR*.

Given the presence of multiple *GR* promoters and their diverse expression patterns in various tissues, it has been hypothesized that these transcripts might be differentially regulated by various environmental stimulus (Ayoubi *et al.* 1996). Thus, we assessed regulation of multiple *GR* promoters in response to cellular signals, including growth factors and potassium-induced depolarization (Figure 9). In this study we found expression of most of first exons to be affected in all tested conditions. Especially, treatment of AtT-20 corticotropic cells with bFGF resulted in strong induction of exon 1.5 transcript and, to a lesser extent, 1.3, 1.4c, 1.6, and 1.7. On the other hand, 30 minutes of N6 cells depolarization led to increased expression of exon 1.4c and 1.11, and decreased levels of exon 1.9. Moreover, our further studies described in Bockmühl *et al.* (2011), show that alternative *GR* transcripts differently influence mRNA stability and translation efficiency. All these findings together reflect a transcriptional and posttranscriptional mechanisms that serve as a tight and tissue- specific control of *GR* expression upon varying physiological demands.

The second part of the thesis provides the evidence for ELS- induced DNA methylation changes at CpG3 within *GR* promoter and up-regulated expression of GR in hypothalamic Crh- releasing neurons (Figure 15). We further demonstrate that observed enhanced *GR* expression translates into higher GR protein transcriptional activity. The latter is shown by increased occupancy of GR at regulatory regions of GR target genes (Figure 19) and their induced expression levels (Figure 16).

Given the fact that almost all identified mouse *GR* first exons lie within an upstream CpG islands, it was hypothesized that DNA methylation at the *GR* promoter might be responsible (at least in part) for regulation of *GR* expression (Bockmühl *et al.* submitted). Extensive methylation analysis of the whole mouse *GR* promoter, covering 3.4 kb of the untranslated exonic region, revealed only sparse methylation (Bockmühl *et al.* submitted). These observations are consistent with the concept of methylation-free promoters associated with CGIs (Deaton *et al.* 2011). In contrast, upstream of exon 1.3 at the CGI shore, moderate levels of DNA methylation were detected. Bisulfite sequencing revealed hypermethylation of a specific CpG residue (CpG3) in adult mice with a history of ELS. Computational analysis predicted the differentially methylated CpG to be part of a putative binding site for the multifunctional transcription factor Yin Yang 1 (YY1) (Bockmühl *et. al.* submitted). This site is homologues to a previously identified distal YY1 binding site (dYY1) within the human *GR* (Breslin *et al.* 1998).

It is important to note, that one study (Weaver *et al.* 2004) reported relatively high level of methylation at the investigated 1.7 rat *GR* promoter region (~15-100%). Their findings were so far only partly reproduced by other researches. For example, MS applied at PND2-PND14 in Sprague Dawley rats has been shown to increase plasma corticosterone release and behavioral abnormalities. However, the authors detected unaltered methylation at the *GR* 1.7

promoter (Daniels *et al.* 2009). In recent post mortem studies, despite decreased level of 1F transcript in the hippocampus of MDD patients, the authors did not find differential methylation of the *GR* 1F promoter, probably due to the overall low methylation level. Importantly, total *GR* mRNA expression was unchanged (Alt *et al.* 2010). In addition extensive methylation analysis of the mouse *GR* promoter performed in our laboratory revealed almost no methylation at 1.7 (Bockmühl *et al.* submitted). These discrepancies between studies may result from strain-specific differences (Long-Evans vs Sprague Dawley), species-specificity (human versus rat versus mouse) and different type of stressors experienced (MS versus low or high maternal care).

Therefore, to prove that our previously reported methylation results are not strain specific (i.e.; confined to C57BL/6N mice), we analysed the methylation status at the YY1 binding site within the CGI shore region of the *GR* promoter in the hypothalamus, hippocampus and blood of CD1 mouse and compared to C57BL/6N. Indeed, the data provide evidence indicating a conserved methylation pattern at the region of interest. Additionally, we observed a similar methylation pattern among all tissues tested (Figure 11), suggesting a conserved methylation at the YY1 binding site across tissues.

Given the high homology between the mouse and human *GR* promoter, we aimed to study whether methylation at the distal YY1 binding site occurs in human as well. In fact, we found methylation in various human neuroblastoma cell lines, cells from buccal swabs, and blood drowns as well as in post mortem brain samples. These findings indicate a potential regulation of the human *GR* by YY1 and might serve as a start point for the comparison of methylation patterns in tissues from control subjects and patients with stress-related brain disorders. However, due to the limited access to human brain tissues, we were unable to follow-up this question.

However, several studies have reported that alterations in epigenetic marks of genes previously implicated in development of brain disorders might be detectable across peripheral tissues and can distinguish patients from controls (Dempster *et al.* 2011; Heyn *et al.* 2012; Masliah *et al.* 2013). These results suggest that peripheral blood cells might serve as a clinically valuable surrogate for brain tissue, given the easy access to this source in patients. Importantly, as shown in Figure 11 and 13, we identified concordant methylation patterns at the *GR* promoter between brain and blood, both in human and mouse. Consequently, it would be of interest to investigate the methylation pattern at the *GR* promoter in the blood of ELS mice. In case of peripheral methylation changes in mice, the same region of the human GR promoter could be assessed in the blood cells of depressed patients with a history of childhood adversity. Changes in *GR* promoter methylation in peripheral blood cells might resemble changes occurring in the brain and can serve as a potential easy accessible and non-invasive molecular biomarker of altered GR functioning in relation to specific psychiatric diseases following a previous stress history.

As already mentioned, corticosteroids which are on one side essential for brain function, can also lead to development of pathological states within brain structure and function (de Kloet *et al.* 2005). This effect highly depends on the timing and duration of the exposure, and previous history of stress (Lupien *et al.* 2009). One of the most vulnerable time points in life is the early postnatal period, characterized by a high plasticity of the developing brain, and its sensitivity for programming and mal-programming by endogenous and exogenous factors (Herman *et al.* 2013; de Kloet *et al.* 2005). Therefore, adverse life events experienced during this period are thought to induce long-lasting changes in the endocrine and behavioral phenotype (Andersen *et al.* 2003). In rodents, disruption of the pup-mother relationship is one of the most potent early-life stressors and can lead to the development of a vulnerable phenotype with an increased reactivity to stress in adulthood (de Kloet *et al.* 2005; Lupien *et*

al. 2009). In fact, as shown previously in our laboratory, mice with a history of MS display in adulthood depression-like symptoms such as an enduring dysregulation of the HPA axis, including increased basal secretion of GCs, hyperreactivity to an acute stress applied later in life, and an impaired response to the dexamethasone suppression test (Murgatroyd *et al.* 2009), indicating an impaired GC-mediated negative feedback. The latter is suggested to occur due to a reduced function or expression of GR within tissues involved in HPA-axis regulation (Zunszain *et al.* 2011; Carvalho *et al.* 2008). This is possibly caused by an altered function of the glucocorticoid receptor (Anacker *et al.* 2011), which is supported by the results of the dexamethasone suppression test (DST), showing inappropriate inhibition of cortisol secretion in depressed patients (Holsboer *et al.* 2000). Indeed, previous work in our lab revealed decreased responsiveness to dex application in ELS mice, as compared to controls, suggesting reduced negative feedback efficiency (Murgatroyd *et al.* 2009). Surprisingly, the same mice do not show a down-regulation of *GR* mRNA expression at any level involved in the regulation of the HPA axis. Our present study revealed actually an up-regulation of *GR,* the effect restricted to in the hypothalamic PVN (Bockmühl *et al.* submitted). This finding is distinct to current knowledge regarding *GR* expression in the brain of individuals with a history of early life adversity. For example, decreased hippocampal *GR* expression was reported in suicide victims who experienced childhood abuse in comparison to controls and suicide victims without a history of ELS (Labonte *et al.* 2012; McGowan *et al.* 2009). Moreover, adult rat offspring receiving low maternal care show decreased hippocampal *GR* expression and reduced glucocorticoid feedback sensitivity in comparison to offspring of high-caring mothers (Liu *et al.* 1997). These discrepancies between studies could be related to species-specificity between human, rat and mouse, or types of experienced stressor (maternal separation, quality of maternal care and childhood abuse), however further studies would be necessary to clarify this matter,

Numerous studies in human and rodents indicate that hyperactivity of the hypothalamus–pituitary–adrenal axis and consequently increased levels of glucocorticoid hormones occur due to impaired GR-mediated negative feedback. Given our previous results about regulation of *GR* first exons by various signaling pathways (Figure 9), it was examined whether expression of specific transcripts is modulated by ELS. We observed up-regulation of multiple transcripts in the hypothalamic PVNs of 3 month old mice with a history of maternal separation (Figure 14). This finding is consistent with our previous observation of increased total *GR* mRNA (Bockmühl *et al.* submitted) and protein levels (Figure 15a). Up-regulation of the major transcripts 1.6 and 1.11 contributed in particular to an increase in total *GR* mRNA. It is important to note that exon 1.7, previously reported by Weaver *et al.* (2004) to be responsive to the quality of maternal care was not altered. Together, these results indicate that in mice multiple *GR* promoter transcript respond to environmental signals induced by postnatal stress exposure when compared to rat.

Given the fact that alternative *GR* first exons differ in their RNA stability and translation efficiency (Bockmühl *et al.* 2011), it raised the question of whether enhanced *GR* mRNA levels translate into higher GR protein levels. Indeed, our IHC results indicate elevated GR protein levels exclusively in PVN of ELS mice (Figure 15).

In order to assess the biological relevance of increased total *GR* mRNA and GR protein expression in the PVN of MS mice, transcriptional activity of GR was tested. Indeed, quantitative RT-PCR analysis demonstrated stronger induction of some GR downstream target genes in ELS vs. control mice, following intraperitoneal corticosterone injection. Genes containing in their sequence up to two glucocorticoid response elements (GREs), i.e. *Fkbp5, Sgk1, and Dusp1* (Arteaga *et al.* 2008; Hubler *et al.* 2004; Tchen *et al.* 2010), showed a differential regulation in ELS mice, suggesting that their inducibility may be facilitated when GR expression is increased. Importantly, we did not observe enhanced GR-dependent

expression of *Fkbp5* and *Sgk1* in those tissues where *GR* expression was unaltered, i.e.; the hippocampal DG (Figure 17). Moreover, we could support improved GR signaling in the PVN by higher GR occupancy at the intronic GRE site of *Fkbp5* and at one of the two sites of *Dusp1* (GRR-29), as demonstrated by *in vivo* ChIP experiments on chromatin isolated from the PVN of corticosterone-treated ELS mice (Figure 19). In the case of *Sgk1*, we did not detect any significant differences in GR occupancy at the GRE located distally from the TSS between ELS mice and controls. This might be explained by the fact that *Sgk1* belongs to the group of immediate early genes and its transcription occurs rapidly (> 20 minutes) in response to various cellular signals (Lang *et al.* 2010). Hence, GR might be partly released from the *Sgk1* GRE, while differences on mRNA level are still present. In the DG GR occupancy at the *Fkbp5* and *Sgk1* genes was unchanged (Figure 20).

Importantly, in the present work we detected GR expression to be confined only to Crh-producing neurons, which are important regulators of the HPA axis (Figure 15b and (de Souza *et al.* 2010). Moreover, the direct action of GR on *Crh* is supported by the *in vivo* ChIP data, showing upon intraperitonal corticosterone application GR binding to the negative GRE (nGRE) localized at the *Crh* promoter (Bockmühl *et al.* submitted). On the other hand, Avp and GR do not co-localize in the PVN arguing against a cell-autonomous mechanism (Figure 15b). Lack of co-localisation between GR and Avp might explain the inability of glucocorticoids to suppress *Avp* expression in ELS mice [Figures 15b and 18, and (Liposits *et al.* 1987)]. Conversely, this lack of GR expression in Avp positive neurons might make *Avp* a particular suitable target for ELS dependent epigenetic programming.

One of the possible explanations for the physiological function of up-regulated *GR* in ELS mice might be a compensatory response to increased *Avp* mRNA in the PVN. This view is supported by the delayed increases of hypothalamic *GR* levels in mice with a history of maternal separation. Changes in *GR* expression become evident in early adulthood (6 weeks

of life) (Bockmühl *et al.* submitted), whereas changes in *Avp* mRNA expression, as shown by Murgatroyd *et al.* (2009), appear directly after exposure to MS (PND10). Summarising, up-regulation of *GR* in the PVN of ELS mice, is proposed to serve as an adaptation to a hyperactive HPA axis and, by increased signalling, it might compensate for reduced GR efficiency at other levels unidentified so far.

Chronic stress in adulthood is another risk factor for the development of stress-related psychiatric disorders (Lupien *et al.* 2009). However, stress reactivity in adulthood may be modulated by previous stress experience, including early-life stress (de Kloet *et al.* 2005). Importantly, recent studies in our laboratory (as shown in Bockmühl *et al.* submitted) suggest that postnatal exposure to ELS attenuates the deleterious consequences of adult chronic unpredictable stress (CUS), when compared to mice undergoing just one of these stress exposures (either ELS or CUS). The behavioural, endocrine and expression data (collected in Table 11) indicate that ELS x CUS mice cope better with certain adult stressors. That fact might be explained, at least partly, by ELS-mediated up-regulation of *GR* in the crh-positive neurons of the PVN. Altogether, the set of data presented here clearly indicates that elevated GR protein levels in the PVN result in increased GR signalling in mice with a history of MS and improved negative feedback regulation, when mice are exposured additionally to CUS during adulthood.

Parameter mesaured	ELS	CUS	ELSxCUS
GR	↑	↔	↑
Avp	↑	↔	↑
Crh	↔	↑	↔
HPA axis function following acute stress	↑	↑	↔
GC feedback efficacy	↓	↓	↑

Depression-like behavior	↑	↑	↑
Anxiety-like behavior	↔	↑	↔
Spatial learning	↓	↓	↑

Table 11: Synopsis of behavioural, endocrine, and expression data from Bockmühl *et al.* submitted
The behavioural, endocrine, and expression data of adult male mice after exposure to ELS, CUS or combined ELS x CUS.

Altogether, these data suggest that ELS-dependent enhanced *GR* expression confers higher repression to the *Crh* gene and restrains CUS-induced *Crh* mRNA expression. Consequently, this might lead to normalised HPA axis responsiveness following acute stress, more efficient negative feedback inhibition and improvement of behavioural phenotypes induced by CUS (Bockmühl *et al.* submitted, Table 11).

Conclusion

Summarizing, the results presented in this thesis, together with our previous studies, indicate that early life adversity influences brain functions later in life by alterations in HPA-axis activity. We report critical epigenetic changes at the *GR* promoter, which open the opportunity to better understand the molecular basis of programming and mal-programming in response to environmental signals and their contribution to the development of stress-related psychiatric disorders.

Moreover, conservation of CpG methylation within the YY1 binding site across neuronal and peripheral tissues in different mouse strains and human, strongly indicate a conserved mechanism of YY1-dependent regulation of human *GR* gene expression and warrant further investigations into this topic under different stress-related disease conditions.

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Acknowledgements

During my PhD I have been surrounded by many friendly and supportive people, without them these years would have been much harder, therefore I am thankful to all of them.

First of all I would like to thank my supervisors Dr. Dietmar Spengler: for giving me the opportunity to accomplish this PhD, his scientific supervision and advices; and Dr. Carsten Wotjak for all his support and for taking over my supervision at the LMU.

I am really thankful to Dr. Yvonne Bockmühl for helping me throughout my PhD by sharing her knowledge and experience.

I would like to thank all the members of the research group Spengler: Marc Bettscheider, Dr. Yvonne Bockmühl, Dr. Guillaume Daniel, Mareen Engel, Anke Hoffmann, Stella Iurato, Yannick Menger, Dr. Chris Murgatroyd, Dr. Udo Schmidt-Edelkraut, Dr. Yonghe Wu and Christoph Zimmermann for their help in the lab, good advices and friendship.

I would like also to thank Dr. Osborne Almeida and Dr. Alexandre Patchev for their scientific support.

I thank my friends for the good times spent together and having shared the joys and sorrows of this PhD.

Above all, I thank my husband Michal and our daughter Kaja for their patience, understanding, motivation and belief in me. Without them I would have not been able to face all the difficulties.

Finally, I thank my family for the support through all these years and help in taking care of Kaja during writing the thesis. I know you are proud of me.

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Declaration / Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet. Weiterhin erkläre ich, dass diese Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt wurde. Auch versichere ich, dass ich mich nicht anderweitig ohne Erfolg einer Doktorprüfung unterzogen habe.

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