Chapter One

General introduction

1.1 Corticosteroids and the brain

The Hypothalamus-Pituitary Adrenal (HPA) axis

During daily life, the human body is faced with internal and external stimuli (also referred to as stressors) that challenge homeostasis. The body responds to these stimuli by turning on the "stress response" (Karatsoreos and McEwen, 2013), that enables the body to adapt and cope with the situation until the challenge has passed. The stress response in our body is among others regulated by the sympathetic nervous system and the hypothalamo-pituitary-adrenal (HPA-axis). The onset of the HPA-axis is mediated by the limbic brain structures, i.e. prefrontal cortex, amygdala and the hippocampus, which together form the interface between the incoming sensory information and the appraisal process (de Kloet et al., 2005). When homeostasis is threatened, this results in a release of catecholamines from sympathetic nerves and the adrenal medulla and the secretion of Corticotropin-Releasing Hormone (CRH) from the hypothalamus. CRH stimulates the synthesis and release of Adrenocorticotropic Hormone (ACTH) from the pituitary which in turn promotes the secretion of glucocorticoids (GC), being cortisol (man) or corticosterone (rodent) from the adrenal cortex (reviewed by McEwen (McEwen, 2000)) (Figure 1.1). GC feed back in an inhibitory manner on the pituitary and on the limbic brain structures that have led to the initial activation of the HPA axis (de Kloet et al., 2005).

The hippocampus is one of the limbic brain regions where GC are able to exert a negative feedback action, due to the high concentrations of GC receptors that are expressed there (Chao et al., 1989; Reul and de Kloet, 1985; van Steensel B. et al., 1996). The hippocampus plays an important role in learning, memory consolidation, mood and regulation of the stress response. The hippocampus contains the cell fields Cornu Ammonis (regions CA1 through CA4) and Dentate Gyrus (DG) which together form an internal trisynaptic circuit intended for hippocampal information processing. Hippocampal neurons display a high degree of structural and functional plasticity during exposure to acute and chronic stressors, which is in part modulated by GC via binding to their receptors.

Acute and Chronic stress

A single challenge of the body's homeostasis, such as the acute stressor of a thesis defense, results in secretion of cortisol (CORT) from the adrenal cortex in the blood circulation. This surge of CORT reaches all tissues including the brain and is aimed to restrain the initial stress reaction. CORT promotes adaptation to the stressful situation and restores homeostasis. This process of adaptation to change to restore homeostasis is called allostasis. In case of prolonged exposure to the stressor, a state of "chronic stress" may develop in which patterns of secretion change and exposure

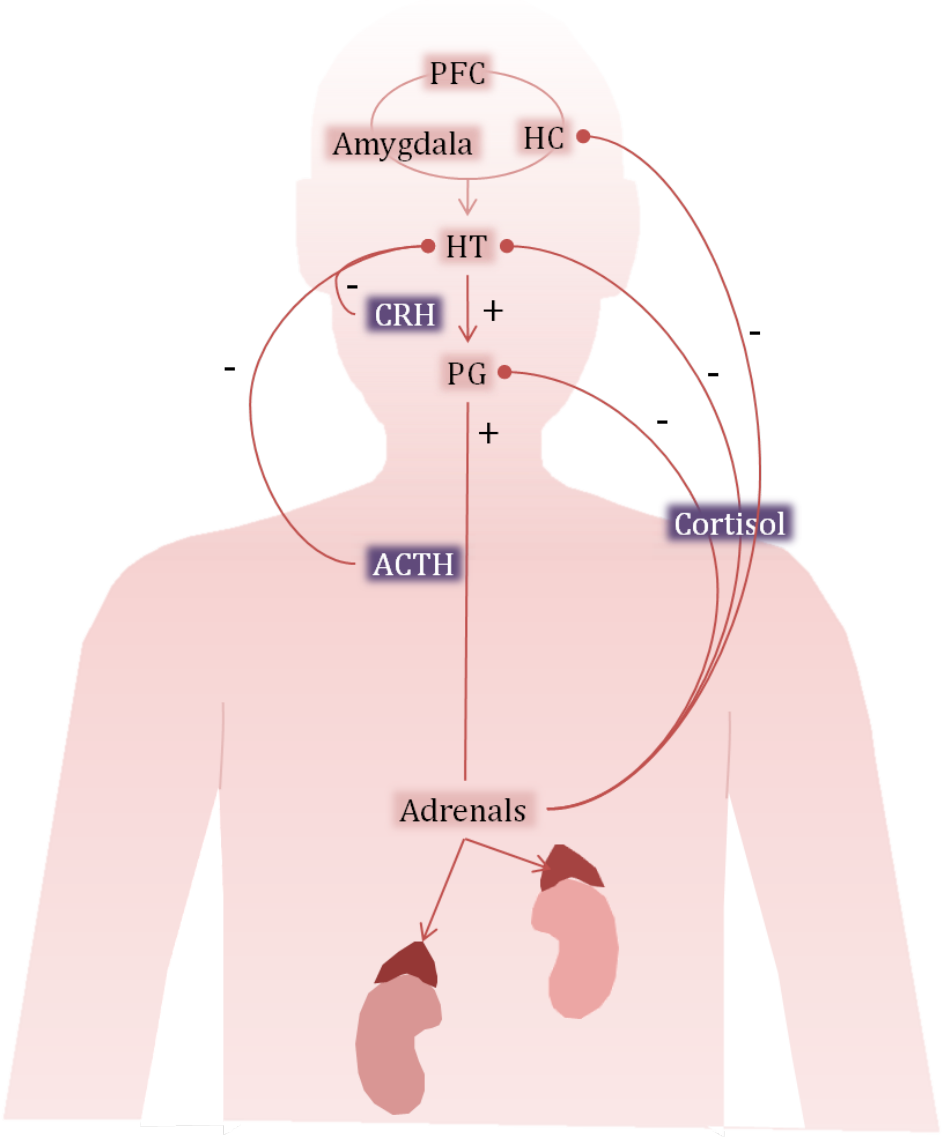

Figure 1.1: The HPA-axis.
Graphic illustration of the Hypothalamus-pituitary-adrenal (HPA) axis. PFC: Prefrontal Cortex, HC: Hippocampus, HT: Hypothalamus, PG: Pituitary Gland

of brain and body to high CORT becomes prolonged. Since CORT also generates energy substrates to cope with a stressor, chronic stress has an increasing cost, also called allostatic load. If such a condition lasts, adaptation is compromised and the organism becomes more vulnerable to develop *e.g.* cardiovascular, metabolic disease and stress-related neuropsychiatric disorders such as major depressive disor-

der or post traumatic stress syndrome. The circumstances during which this shift to impaired adaptation occurs depends among others on the genetic background of the organism and acquired strategies to maximize efficient energy expenditure and adaptation to limit overdrive of the stress reaction (Herman, 2013). The ability of an organism "to cope with environmental tumult by bending and not by breaking" is also referred to by the term "resilience" (Karatsoreos and McEwen, 2013).

Chronic stress affects brain regions at the cellular level as for instance is the case with the hippocampus that is affected structurally and functionally by chronic stress. This is among others reflected in suppression of neurogenesis in the subgranular zone of the dentate gyrus (Heine et al., 2004; Magarinos et al., 1996; Gould et al., 1997). Besides causing structural changes, CORT affects electrical properties of hippocampal neurons. Chronic stress or chronic CORT exposure suppresses hippocampal long term potentiation (LTP), a lasting synaptic strengthening that likely underlies learning and memory formation (Alfarez et al., 2003; Bodnoff et al., 1995; Krugers et al., 2006).

A dual receptor system modulates glucocorticoid action

CORT circulates in the blood where $95\,\%$ is bound to corticosteroid binding globulin (CBG) or albumin which prevents CORT from entering the brain (Pardridge and Mietus, 1979; Moisan et al., 2014). The remaining $5\,\%$ unbound CORT passes the blood-brain barrier and binds to two types of receptors in the brain: the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (Reul and de Kloet, 1985). These receptors belong to the superfamily of ligand-activated nuclear receptors and have a distinct localization in the brain. MR is predominantly localized in neurons of limbic structures, while GR is expressed in every cell, albeit at different levels. Stress centers in the brain, such as hippocampus, amygdala and hypothalamus express high amounts of GR (Morimoto et al., 1996). The balanced activation of MR and GR is an important determinant of neuronal excitability, neuronal health and stress responsiveness (de Kloet et al., 1998; de Kloet et al., 2005).

MR is abundantly expressed in the limbic brain structures amygdala and hippocampus (Reul and de Kloet, 1985). This implies that the receptor plays a role in the functionality of these brain areas. Amygdala circuits are important for emotional expressions such as fear and anxiety, while the hippocampal structure provides a context to these emotional reactions in time and place. MR has a high affinity for CORT ($K_d = 0.5\,\text{nM}$), ~10 fold higher than GR ($K_d = 2.5\text{–}5\,\text{nM}$), and as a result MR is activated during lower, basal concentrations of circulating CORT (Figure 1.2) (Reul and de Kloet, 1985). Activated MR is considered to be important for the tone of the HPA axis, also described as the threshold or sensitivity of the stress response system (de Kloet et al., 1998).

Via MR, CORT exerts both genomic and non-genomic actions. Whereas the non-genomic actions occurring with a delay of seconds to minutes are considered to be responsible for fast CORT effects, the genomic effect is slower and long-lasting.

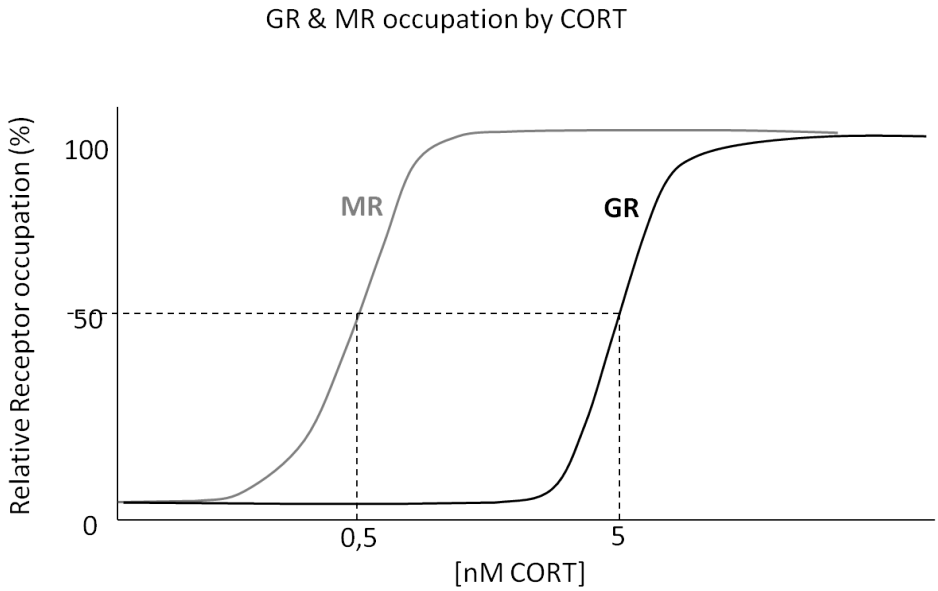

Figure 1.2: Nuclear Corticosteroid Receptor occupancy by CORT.
Simplified graph representing the relative nuclear MR and GR occupation by CORT. MR has a higher affinity for CORT and becomes occupied at lower concentrations of CORT in comparison to GR (Reul and de Kloet, 1985).

Genomic CORT effects can be detected at 15 – 60 minutes after stress exposure and may last for several hours. Knowledge regarding the actions of genomic MR on the cell has been available for decades, although knowledge on the target genes of MR remains sparse. The features of non-genomic MR-mediated effects of CORT have only recently been uncovered (de Kloet et al., 1998; de Kloet et al., 2005). GR has a lower affinity for CORT in comparison with MR and consequently GR only becomes occupied during elevated concentrations of circulating CORT (Figure 1.2).

Elevated CORT is triggered during the stress reaction and is also observed at the circadian and ultradian peaks (Stavreva et al., 2009). Similar to MR, GR can mediate genomic effects of CORT that modulate the transcription of CORT-responsive genes (Oakley and Cidlowski, 2013). In addition, GR was found to mediate non-genomic actions in the lateral amygdala and medial prefrontal cortex (mPFC), where it is indicated to play a role in memory consolidation (Barsegyan et al., 2010; Johnson et al., 2005).

GR and MR mediate complementary and different, sometimes opposing, actions of CORT. While MR is involved in maintenance of neuronal excitability and basal activity of the stress system and onset of the stress reaction, GR activation results in subsequent suppression of excitability transiently raised by excitatory stimuli, recovery from stress and behavioral adaptation. Their balanced activation is an important determinant of neuronal excitability, neuronal health and stress respon-

siveness (de Kloet et al., 1998; de Kloet et al., 2005) In the current thesis, we aim at gaining more understanding of the interaction of MR and GR with the genome. Because of its role in the normalization of the stress response and how this might be impaired in coping with chronic stress, the GR has received the most attention in the research described in this thesis.

1.2 Glucocorticoid Receptor

The GR protein is composed of three major domains, namely an N-terminal transactivation domain (NTD), a DNA-binding domain (DBD) and a C-terminal ligand binding domain (LBD) (Figure 1.3) (Reviewed in (Oakley and Cidlowski, 2013)). Whereas the DBD contains motifs that recognize and bind target DNA sequences, the LBD interacts with the ligand and contains an Activation Function (AF2) that interacts with coregulators in a ligand-dependent manner. In addition, the LBD aids in dimerization of GR, a topic which will be described in the following sections (Bledsoe et al., 2002). The NTD contains a second transcriptional activation function (AF1) that is able to interact with coregulators and the basal transcription machinery. The NTD is the primary site for post-translational modifications such as phosphorylation (P), sumoylation (S), ubiquitination (U) and acetylation (A) (Figure 1.3).

The human GR gene is composed of 9 exons, which due to alternative splicing and alternative translation initiation mechanisms can result in various GR-subtypes, namely GRα, GRβ, GRγ, GR-P and GR-A (Figure 1.4) (Reviewed in (Oakley and Cidlowski, 2013)). GRα is the most abundant variant and is known as the classical GR-protein which is able to mediate the genomic actions of glucocorticoids. The second GR-variant, GRβ, has a disrupted LBD and functions independent of ligand as a dominant negative inhibitor of GRα on many glucocorticoid-responsive target genes (Yudt et al., 2003; Oakley et al., 1996; Charmandari et al., 2005). However, the expression of GRβ is low in the human hippocampus (De Rijk et al., 2003). The third variant, GRγ, is widely expressed, albeit at low levels, and binds glucocorticoids and DNA in a manner similar as GRα (Thomas-Chollier et al., 2013). However, GRγ is impaired in its ability to regulate glucocorticoid-responsive reporter genes due to a disrupted DBD and therefore has different DNA-targets in comparison to GRα. Both GR-A and GR-P isoforms miss large regions of the LBD and are not able to bind CORT. The functional role of GR-A and GR-P is less clear, but there are indications that GR-P modulates the transcriptional activity of GRα in a cell-type specific manner (de Lange P. et al., 2001).

The GR-transcripts can be alternatively translated into a subset of GR-protein isoforms. GRα mRNA, the GR-subtype which is known to interact with the genome after being activated by GC, can be translated into 8 protein isoforms: GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3 (Lu and Cidlowski,

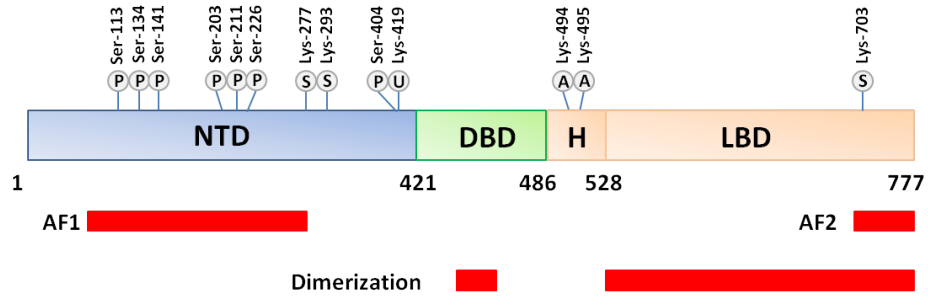

Figure 1.3: GR structure and post-translational modification.
Shown are the N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a C-terminal ligand binding domain (LBD), transcriptional activation function (AF1) and Activation Function (AF2). H (Hinge region). *Adapted from: Oakley et al, J Allergy Clin Immunol. 2013 Nov; 132(5): 1033–1044.*

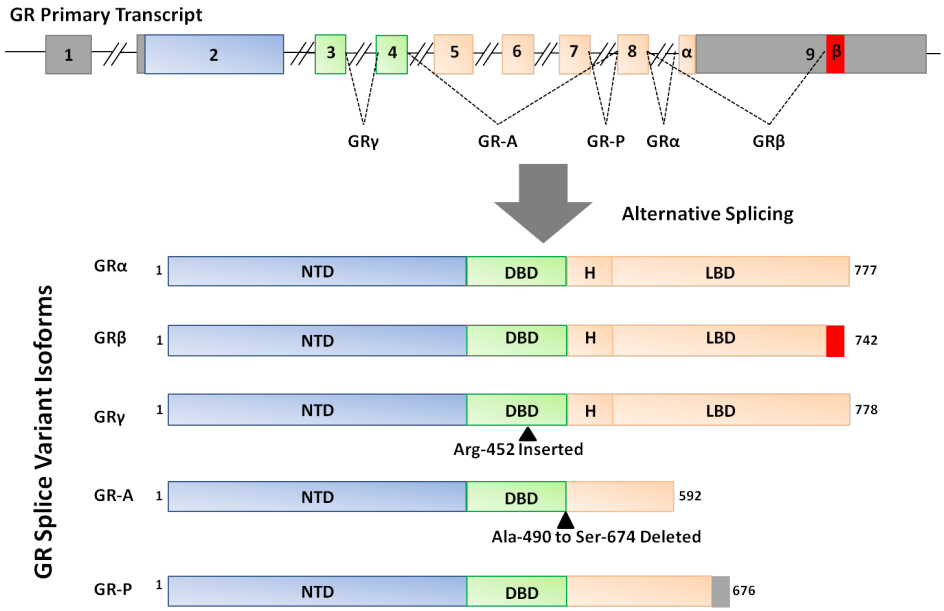

Figure 1.4: GR splice variants.
Alternative splicing events result in the expression of GRβ, GRγ, GR-A, and GR-P. *Adapted from: Oakley et al, J Allergy Clin Immunol. 2013 Nov; 132(5): 1033–1044.*

2005)(Reviewed in (Oakley and Cidlowski, 2013; Yudt and Cidlowski, 2002). These isoforms differ in their NTD-length, which is attributed to 8 AUG codons that are present in exon 2. Since the mRNA transcripts corresponding with GRβ, GRγ, GR-P and GR-A contain exon 2 as well, it is expected that these proteins will have isoforms that are comparable to GRα. The 8 GRα isoforms are conserved through species including rat, are all sensitive to GC and are able to bind GRE's. However, they differ in their intracellular localization when the hormone is absent, are expressed in a

celltype-dependent manner and they have distinct gene transcriptional profiles (Lu and Cidlowski, 2005). What the mechanism behind the isoform translation exactly is and how this differs per celltype, is not yet understood. The antibody used in the current thesis involves H300 (Santa Cruz), which recognizes amino acids 121–420 of human GR and will recognize the GRα-A, GRα-B and the GRα-C isoforms. These isoforms are localized in the cytoplasm of cells in the absence of hormone and translocate to the nucleus on glucocorticoid binding. In the remaining text of this thesis, GR is synonomous for these 3 types of isoforms, unless stated otherwise.

Inactive GR resides in the cytoplasm where it is part of a Heat Shock Protein 90 (HSP90) chaperone complex that prevents GR from being degraded (Pratt and Toft, 1997; Vandevyver et al., 2012). These chaperones include FK506 binding protein 51 (FKBP51) which operates in an ultrashort feedback loop in the control of GR activity (Vermeer et al., 2003). When CORT is present in the circulation, it can diffuse freely through the cell membrane into the cytoplasm where it is able to interact with a variety of proteins. When the concentration of CORT is high enough, GR will bind CORT at its ligand-binding domain (LBD) (Reul and de Kloet, 1985; Spiga and Lightman, 2009). In this activated state, GR can be phosphorylated by several kinases including cyclin-dependent kinases (CDKs), c-Jun N-terminal kinase (JNK) and mitogen-activated kinases (MAPKs). The phosphorylation of GR can either inhibit or facilitate the attraction of cofactors to GR that can modulate its transcriptional activity (Chrousos and Kino, 2009; Galliher-Beckley and Cidlowski, 2009).

After CORT-binding, activated GR translocates to the nucleus using the cytoskeleton facilitated by HSP90 that has a fundamental role in promoting GR nuclear mobility (Reviewed in (Vandevyver et al., 2012).) In addition, there are two nuclear localization signals (NLS) present in the GR protein: NLS1 and NLS2, which are located near the DBD-hinge boundary and within the LBD respectively. These signals are recognized by importins which mediate the nuclear transport of GR as well. Within the nucleus, GR can interact with the genome and other proteins to regulate the transcription of genes. These interactions can be subdivided into two main processes: transactivation and transrepression (Figure 1.5). These two modes of genomic interaction by GR appear to be context-dependen and proceed directly with DNA (transactivation) or indirectly via protein-protein interaction .

Transactivation: Glucocorticoid Response Elements (GRE)

During the process of transactivation, GR binds as a homodimer directly to fifteen-nucleotide-long sequences known as Glucocorticoid Response Elements (GREs). Here, the GR undergoes conformational changes that result in the recruitment of coregulators and chromatin-remodeling complexes that mediate gene transcription. Well-known coactivators include steroid receptor coactivators (SRC 1–3) and histone acetyltransferases CBP/p300 (Zalachoras et al., 2013). This interaction is considered to induce the transcription of the gene that is associated with the GRE. In this thesis we consider the genes that have their transcription start site nearest to

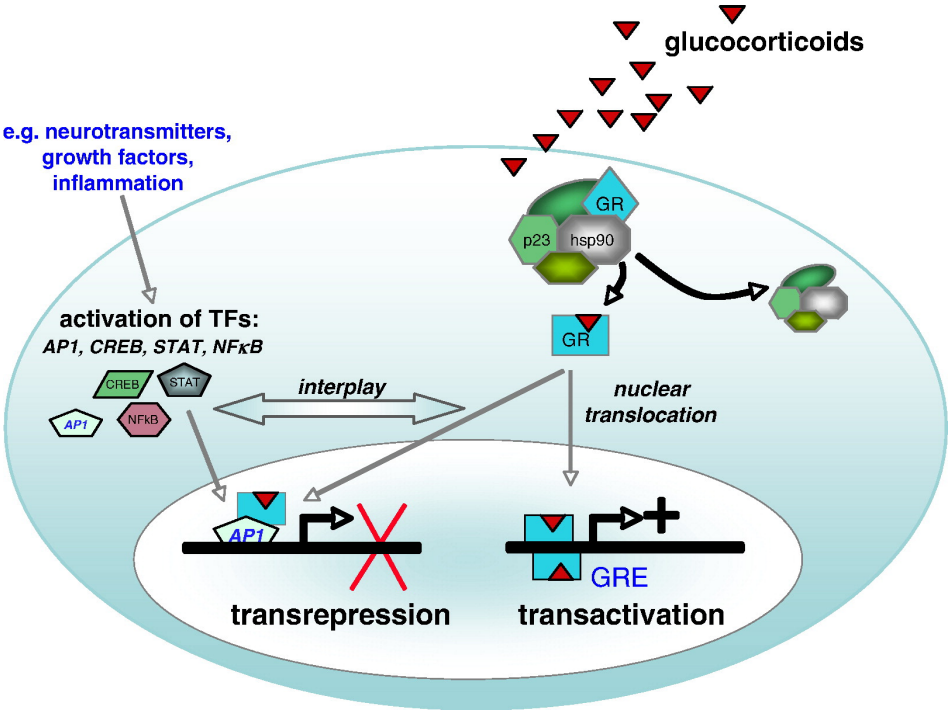

Figure 1.5: Genomic interactions of MR and GR.
The two main interactions that GR can have with the genome: transactivation and transrepression. GR can interact as a dimer with specific 15 nucleotide sequences (GREs or nGRE) as is shown on the right and is called transactivation. The left side represents an indirect protein-protein interaction of GR with the genome via interaction with other transcription factors, called in the literature transrepression. The result of this interaction is that the transcriptional activity of the bound transcription factor is impaired, which is the mechanistic underpinning of the physiological role of glucocorticoids in dampening the initial reaction to stressors. (Reprinted with permission from N.A. Datson.)

the GRE to be GRE-associated and therefore potentially transcriptionally regulated by GR binding to the GRE. However this is not always the case. GREs are known to be localized within promoter-regions of GR-target genes, but recent evidence, including the research described in this thesis, has shown that GREs can be located within introns, exons and intergenic regions at long distances from the nearest gene as well. In the end, whether binding of GR to a GRE is functional has to be demonstrated by differential expression of the associated genes.

A GRE consists of two inverted hexameric half-site motifs separated by three base pairs (Strahle et al., 1987). In 1989, Beato et al published the consensus-sequence for a GRE being 5′-GGTACAnnnTGTTCT-3′, but later research has shown that this GRE-sequence is not as static as presented here (Beato et al., 1989). *In vitro* experiments have shown that GRE composition can differentially affect GR conformation and regulatory activity, indicating that the GRE itself tailors the activity of the receptor towards target genes (Meijsing et al., 2009). How exactly this is achieved *in vivo* remains to be elucidated. Since MR is very similar to GR, it is be-

lieved that it is likely to bind to similar sequences at the DNA. However, this needs more investigation.

There are a number of well-known GR-targets that have been linked to a described functional GRE. An example is Period 1 (Per1) which is a protein that is important for the maintenance of circadian rhythms in the brain and is expressed in the rat hippocampus (Conway-Campbell et al., 2010). In the hippocampus, ultradian pulses of injected CORT in adrenalectomized rats have shown to be consecutively followed by 1) nuclear localization of GR in the hippocampus, 2) GR cycling on the GRE-containing Per1 promoter, 3) pulses of heteronuclear RNA (hnRNA) expression of Per1 and 4) accumulation of Per1 mRNA during several hours of treatment reaching a plateau that is maintained by the hourly pulses (Conway-Campbell et al., 2010). When the hourly pulses of CORT decline, Per1 regulation responds accordingly.

Kruppel-like factor 9 (KLF9) is another example of a GRE-containing gene. Klf9 is a transcription factor implicated in neuronal development and plasticity and its mRNA expression is increased in the mouse hippocampus after CORT injection (Bagamasbad et al., 2012). Two GR/MR response elements have been identified upstream of the Klf9 gene and both were shown to be functional. Other genes with documented GREs are CORT-responsive genes Metallothionein 2A (MT2a) (Kelly et al., 1997), Glucocorticoid-Induced Leucine Zipper (GILZ) (Cannarile et al., 2001) and DNA-damage-inducible Transcript 4 (Ddit4) (Datson et al., 2011; So et al., 2007) which will receive more attention in the current thesis.

Identification of functional GREs in the genome is complicated by several factors. In essence, the chromatin should be accessible in order for GR to bind to its targets, which is highly context dependent (Reddy et al., 2012). Factors such as GRE-accessibility and expressed coregulators differ between cell types and therefore provide a different cellular context that determines the cell type-specific stress response (John et al., 2011; Reddy et al., 2009; Yu et al., 2010). Furthermore, GR-GRE interactions are dynamic, with GR hopping on and off the genome to influence gene transcription (Stavreva et al., 2009). This illustrates the challenges that exist in genomic GR-target identification and the difficulties that lie in proper interpretation of new data.

Negative GREs (nGRE)

Besides activating transcription of target genes, direct binding of GR to GREs can also result in a negative regulation of the corresponding gene. However, there is a slightly different composition of the sequence necessary to achieve this and therefore this sequence is referred to as negative GRE (nGRE). A consensus sequence for nGRE that has been published is CTCC$(n)_{0-2}$GGAGA where the number of nucleotides located between the two main sequences can vary, ranging from 0–2 nucleotides (Surjit et al., 2011). However this sequence is, similar to the GRE-consensus sequence, not static and the composition differs depending on the nGRE investi-

gated. There are several mechanisms that can explain the inhibitory effect of GR-nGRE interaction. First, it was found that binding of GR to an nGRE promotes the assembly of *cis*-acting GR-SMRT/NCoR repressing complexes (Surjit et al., 2011). Second, evidence was identified *in vitro* that two GR monomers bind nGREs in a reversed repeat orientation with strong negative cooperativity which will result in the presence of monomeric GR at nGREs (Hudson et al., 2013; Surjit et al., 2011). Examples of nGRE-containing genes are POMC (Drouin et al., 1993), Glucose-6-phophatase catalytic subunit gene (G6Pase) (Kooi van der et al., 2005) and Prolactin (Sakai et al., 1988).

Transrepression

GR is able to bind indirectly to the genome via protein-protein interaction with other transcription factors. These sites known as "tethering" GR-binding sites do not contain actual DNA-binding sites for GR itself, but instead contain binding sites for other transcription factors that are bound by GR (Kassel and Herrlich, 2007; Pearce et al., 1998). Since this interaction often results in downregulation of the functional effects of the other transcription factors, these binding sites are also referred to as transrepressive sites. This crosstalk of GR with other transcription factors (TFs) vastly expands the range of GR-control over physiological processes as compared to the classical GRE-driven transcriptional control in simple GREs and it is likely that it underlies the highly context-dependent action of CORT.

Classical transcription factors that interact with GR are Activator Protein 1 (AP-1), cAMP response element-binding protein (CREB) and nuclear factor kappa-b (NFκB) (Figure 1.5) (Pearce et al., 1998; Alboni et al., 2011; Rao et al., 2011; Jonat et al., 1990; De Bosscher K. et al., 2008). NFκB and AP-1 are both well-known for their fundamental role in pro-immunological and pro-inflammatory responses and as such are important pharmacological targets for treating inflammatory disorders. In the brain the capability of NFκB to bind DNA has been described (Unlap and Jope, 1995). NFκB expression is increased in the mouse hippocampus after acute and chronic stress (Djordjevic et al., 2015). DNA-binding by AP-1 has shown to be increased after restraint stress in the hippocampus and CREB is associated with long-term memory formation (Bourtchuladze et al., 1994; Miller et al., 2007). Other examples of transcription factors include Oct1 and Ets1 at composite GREs and Oct-1/2, STAT3 (Langlais et al., 2012), STAT6, SMAD3, 4 and PU.1/Spi-1 at tethering sites (Biola et al., 2000; De Bosscher K. et al., 2006; Gauthier et al., 1993; Imai et al., 1993; Jonat et al., 1990; Kassel and Herrlich, 2007; Schule et al., 1990; Song et al., 1999; Stocklin et al., 1996; Wieland et al., 1991). However, the interaction of GR with these regulators is mainly based on studies describing the immunosuppressive and the tumor suppressor properties of GR (Chebotaev et al., 2007; De Bosscher K. et al., 2008; Glass and Saijo, 2010), while very little is known about crosstalk partners in a neuronal context.

In an attempt to gain understanding of the impact of GR on the entire organism, GR-deficient mice have been developed in the past (Cole et al., 1995). However, these mice die shortly after birth and consequently, other models were developed. For instance, $GR^{dim/dim}$ mutant mice that lack the potential to dimerize and as a consequence cannot modulate gene transcription via GREs but retain the capacity for GR-mediated transrepression were created (Reichardt et al., 1998). Interestingly, these mice are viable and it was concluded that transactivation via GREs does not seem to be essential for viability in mice. Later on this conclusion was nuanced by stating that *in vitro* GR dimer mutants (assumed to be GR monomers) are able to bind specifically to a class of more complex GREs and stimulate transcription. It was postulated that GRs can form concerted multimers in a manner that is independent of the DBD-dimer interface (Adams et al., 2003).

1.3 *De novo* identification of GR-targets

Since GR is involved in many essential processes within the body including immunological- and stress-related processes (Chinenov et al., 2012; de Kloet et al., 2005; Harris et al., 2012; Silverman and Sternberg, 2012; Simoes et al., 2012), research aimed at gaining more insight in GR-functioning has received a lot of focus. A lot of insight in GR function in different contexts or cell types can be obtained by understanding the genes and pathways regulated by GR. While various research groups have been active in this area, it has proven to be a challenge to answer the question "what are the primary GR-targets in the genome?" Below we discuss a variety of methodological approaches that can be used to tackle this question.

Expression profiling

Activation of GR results in GR-binding, either directly or indirectly, to the DNA, thus affecting the expression of associated genes. The altered gene expression can be measured by Real-time Quantitative Polymerase Chain Reaction (RT-qPCR), which is a low to medium throughput methodology especially suitable for analysis of specific genes of interest. However, when research involves the detection of genes that are differentially expressed in a genome-wide manner without prior knowledge or preference, other techniques are available such as RNA microarrays, RNA-sequencing (RNA-Seq) and Serial Analysis of Gene Expression (SAGE) (Datson et al., 2012; Datson et al., 2001b; Reddy et al., 2009).

RNA expression profiling enables the analysis of the cell's transcriptome. This approach has been applied to characterize GR-mediated gene expression in the hippocampus and has increased our understanding of genomic pathways regulated by GR (Datson et al., 2001b). These pathways involve general cellular processes such as energy metabolism, cell cycle and response to oxidative stress, but also genes that are important for neuronal structure and plasticity (Datson et al., 2001b; Datson et

al., 2001a; Datson et al., 2012). However, RNA expression profiling in the brain is challenging due to among others its cellular heterogeneity and small and thus difficult to detect changes in gene expression which are mostly in the range of 10–30 % (Datson et al., 2008). This makes identification of differentially expressed genes as a response to a stressful situation or CORT-injection in brain more difficult.

When characterizing GR-dependent changes in gene expression it is important to bear in mind that not all genes that change their expression in response to GR-activation will represent primary GR-targets. For instance, the time point of gene expression measurement after a stress response or administration of CORT is an important factor. The longer the duration between activation of GR and measuring the transcriptional changes, the less likely it is that these transcriptional changes are primary GR-regulated events but rather secondary or even further downstream events. By using the protein synthesis inhibitor cycloheximide it is possible to rule out all differential expression occurring secondary to the initial GR-binding and thus requiring translation of the primary target (Morsink et al., 2006a). Despite the fact that these approaches have provided enormous insight in GR-function, mRNA analysis remains an indirect measurement to determine the primary genomic GR-targets.

In silico modeling

As mentioned earlier, GR is known to bind to GRE sequences and screening the genome for this sequence is another approach to identify potential primary GR-binding sites in the genome. A genome-wide search for the exact consensus hormone response element (AGAACAnnnTGTTCT) revealed the presence of 565 hits in the entire human genome (Horie-Inoue et al., 2006). When comparing this number with expression profiling data of the rat hippocampus where 203 putative CORT-responsive genes were revealed, a discrepancy is evident (Datson et al., 2001b). Apparently, identifying a GRE sequence that is functional at a given genomic location will not guarantee that GR can bind at that same GRE in all cell types or at all CORT concentrations nor at identical sequences present elsewhere in the genome.

Essentially, the chromatin has to be accessible for GR to allow binding to its genomic target. This accessibility differs per experimental setting, timing and cell type, and also depends on the type and amount of ligand. Furthermore, the sequence surrounding a GRE is of importance for guiding cofactors to assist in the GR-DNA interaction, while the cocktail of expressed and therefore available cofactors may vary per cell-type. Therefore the binding potential of GR to specific DNA binding sites will differ between cell-types. Knowledge that has been acquired in the liver, where GR is abundantly expressed, can by no means be extrapolated to the brain and vice versa. DNA binding sites that are predicted *in silico* or have been identified in other tissues have to be verified in every experimental setting.

Combining expression profiling data with predicted GR-binding sequences gives an extra dimension to the likelihood that *in silico* predicted sites are functional. In

addition, evolutionary conservation appears to be a major predictor of functionality of a subset of transcription factor binding sites (Kunarso et al., 2010). Combining this knowledge, evolutionary conserved GREs can be predicted *in silico* by using a Position Specific Scoring Matrix (PSSM) constructed from known GREs. This PSSM can scan genomic regions surrounding CORT-responsive genes and calculates the likelihood that a given 15 bp-long sequence, the length of a GRE, is in fact a functional GRE. This is considered to be more sensitive and precise than screening the genome for consensus sequences (Stormo, 2000). It has to be noted that *in silico* prediction is based on known GR binding sites and does not consider the fact that other yet undiscovered GR-binding sequences that are distinct from the canonical GR consensus motif might exist as well.

Chromatin Immunoprecipitation (ChIP)

To identify the genomic targets to which GR primarily binds, the most proximal readout is to analyze the actual GR-DNA interaction rather than the altered transcriptome. In the past, binding-assays such as electromobility shift assays, reporter assays and DNase footprinting assays have provided invaluable information regarding transcription factor binding to the DNA and enabled elucidation of DNA recognition sequences to many receptors (Payvar et al., 1983; Hellman and Fried, 2007). Their main drawback however, is that it is difficult to apply them to *in vivo* experimental settings and that they have a low throughput (Vinckevicius and Chakravarti, 2012). The rise of Chromatin Immunoprecipitation (ChIP) has enabled isolation of specific protein-DNA complexes from cells and tissues that can subsequently be used to study interactions of transcription factors and other proteins to the whole genome (Nelson et al., 2006). Briefly, the DNA-protein interactions are fixed using formaldehyde, after which the chromatin is sheared and protein-specific antibodies are used to isolate DNA-fragments to which the protein of interest is bound. Sepharose A-beads will bind the antibody-protein-DNA complexes after which they are reverse crosslinked to release the DNA fragments. The DNA-fragments are purified and can be further analyzed (Figure 1.6). Commonly used downstream methods for further analysis of ChIP DNA fragments are described below.

ChIP-on-chip

The DNA fragments obtained in ChIP can be labeled (with a fluorescent tag) and hybridized to complementary probes that are present on a microarray, a technique also known as ChIP-on-chip (Lemetre and Zhang, 2013; Pillai and Chellappan, 2009). Subsequently, the signals of the labeled fragments are measured and since the signal depends on the amount of target sample that binds to the probes, quantitative calculations can be made when comparisons are made between different samples. The probes with ChIP signals can be mapped back to the genome and as a result, genome-wide protein binding sites can be identified.

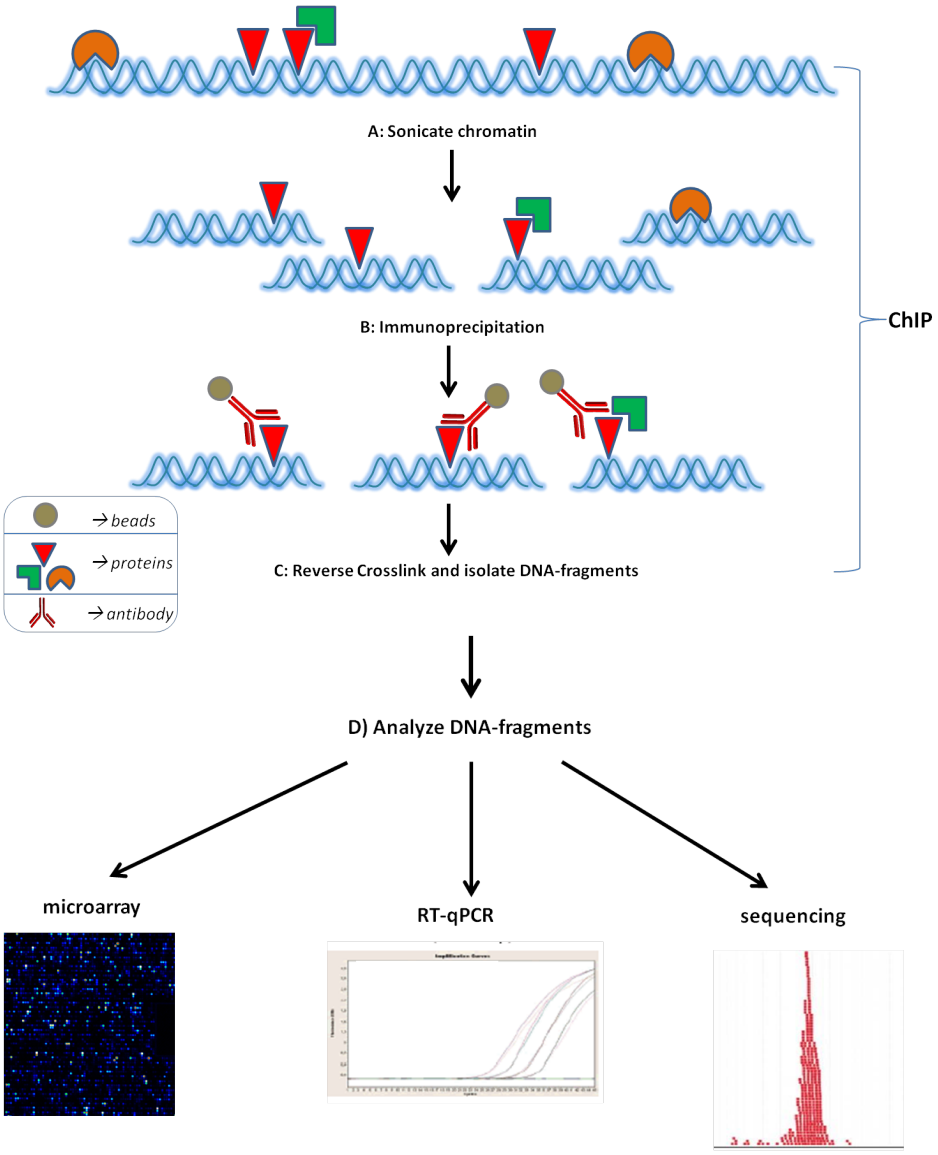

Figure 1.6: Chromatin Immunoprecipitation (ChIP) method.
Graphic illustration representing the basic steps of Chromatin Immunoprecipitation (ChIP). Proteins visualized represent proteins that bind to the DNA, such as transcription factors. A) After crosslinking the DNA-protein complexes, the DNA is sheared by usage of electromagnetic waves, resulting in DNA-fragments. B) By using an antibody specific for the protein of interest, the DNA-fragments to which this protein is bound to, can be selected for isolation. Sepharose-A beads subsequently bind to the antibody-protein-DNA complexes. C) Finally, the complexes are reverse crosslinked and the DNA-fragments are purified. D) The DNA-fragments are now ready for further analysis by Microarray, RT-qPCR and/or sequencing. Here, one sequenced fragment is represented by a red dot. When there is a location highly bound by for instance GR, than it is expected that this region is isolated in a higher amount in comparison to background signals resulting in an overlap of the sequenced fragments (or red dots) visualized by peaks.

The DNA fragments that are present on a microarray are variable and it depends on the research questions that one has which microarray composition is preferred. It is possible for instance to focus on promoter regions (affymetrix_ 900775 _ human) or CpG islands (Weinmann et al., 2002). In addition, there is a possibility to screen only elements that are known to be functional in the human genome, also known as ENCODE regions (agilent_ G4495A-014792_human).

Advantages of using a microarray is that it is easy to use, quantitative, relatively fast and flexible with respect to the genetic components one wishes to analyze. A downside is that it does not allow for identification of ChIP-fragments mapping outside the regions represented on the microarray and to analyze a whole genome of human, rat or mouse, a lot of ChIP-material needs to be available, which is unfortunately often not the case, especially when specific animal tissue, such as hippocampus, is concerned.

ChIP-sequencing

Alternatively, the DNA fragments obtained by ChIP can be analyzed by next-generation sequencing, also known as ChIP-sequencing (ChIP-seq), which allows sequencing of low concentrations of short DNA-fragments in an unbiased manner (Mardis, 2007). Briefly, the DNA-fragments are blunted and ligated to sequencing adapters after which the DNA is amplified using PCR. Subsequently, the DNA-fragments are size-separated on an agarose-gel after which fragments containing an average of 300 base pairs (bp) in length are selected for sequencing. Single-end sequencing of the first $35\,\mathrm{bp}$ of all the fragments results in small sequences, also known as reads, which can be mapped back to the genome to define where they originate from. ChIP-sequencing generates millions of reads and it goes without saying that bioinformatics is essential in data analysis. The amount of generated reads depends on the protein investigated, the experimental model used and the read length. However, according to the guidelines that recently have been established by the ENCODE consortium, the goal is to obtain ≥ 10 million reads for mammalian genomes (Landt et al., 2012).

Compared to ChIP-on-chip, ChIP-seq achieves higher sensitivity and sharper resolution of protein-DNA binding sites, while generating less noise (Euskirchen et al., 2007; Ji et al., 2008; Robertson et al., 2007). There are several high-throughput sequencing platforms available. The Solexa Genome Analyzer was one of the first commercial sequencers and after being purchased by Illumina, the next generation sequencing machines such as the Illumina Genome Analyser were among the most frequently used sequencing platforms. There is various peak calling software available that is used to identify regions of ChIP enrichment, including CisGenome (Ji et al., 2008) and the CLC Genomics Workbench (CLC bio). Researchers who have access to bioinformatics support might choose to use home-made algorithms.. In general, successful experiments identify thousands to tens of thousands of peaks for most transcription factors in mammalian genomes (Landt et al., 2012). In the end,

the choice for specific analysis software is subjective and there is not a standard tool for ChIP-seq analysis.

The options that one has with ChIP-seq seem to be endless. Due to the unbiased approach, binding sites in the whole genome can be identified including intronic, exonic and intergenic regions. This means that one does not need to make a decision at forehand on whether to focus only on promoter-regions, which is the more classic location to focus on in the search for TF-binding sites. Furthermore, there are possibilities to analyze one sample and look at binding sites of multiple proteins to see whether they potentially form protein-complexes on the DNA. Binding peaks can be visualized through means of the UCSC Genome Browser(Kent et al., 2002).

ChIP-seq has been applied in the context of *de novo* primary GR-target identification in several studies. However, these studies were performed in non-neuronal cell cultures, including human lung carcinoma cells (A549), mouse adipocytes (3T3-L1), premalignant breast epithelial cells (MCF10A-Myc), murine mammary epithelial cells (3134) and pituitary (AtT-20) cells (John et al., 2011; Pan et al., 2011; Yu et al., 2010). These studies have contributed to an increased insight in the genome-wide repertoire of GR and the involved molecular mechanisms. Perhaps one of the most striking findings is the low degree of overlap in GR binding sites when comparing different cell types, indicating that GR occupancy of genomic binding sites is highly cell type specific (John et al., 2011). This illustrates the importance of cautiousness when extrapolating data from similar experiments performed in different experimental models and underscores the complex nature of GR signaling.

1.4 Hippocampal plasticity and the mTOR pathway

In the current thesis we have investigated whether the candidate pathway mammalian target of Rapamycin (mTOR) is directly regulated by GR. This pathway is fundamental in regulating neuronal plasticity, which is the structural and functional change in response to a given situation, either physiological or psychological. This is fundamental to hippocampus-dependent learning and memory. The mTOR pathway has been studied extensively in relation to cancer (Akhavan et al., 2010), but knowledge in relation to the hippocampus and to stress is a topic that has gained interest more recently (Liu et al., 2014; Lu et al., 2015; Orlovsky et al., 2014).

The mTOR-pathway is activated by a wide variety of extracellular stimuli such as hormones, growth factors and neurotransmitters (Figure 1.7) and plays an important role in conveying extracellular information to the intracellular environment. The mTOR-pathway exists of two different multiprotein complexes within the cells, mTORC1 and mTORC2, whose activity is initially influenced by neuronal surface receptors and channels such as *N*-methyl-D-aspartate receptors (NMDA-R), brain-derived neurotrophic factor (BDNF) and dopaminergic and metabotropic glutamate receptors (mGluRs) which are fundamental to maintaining long-term potenti-

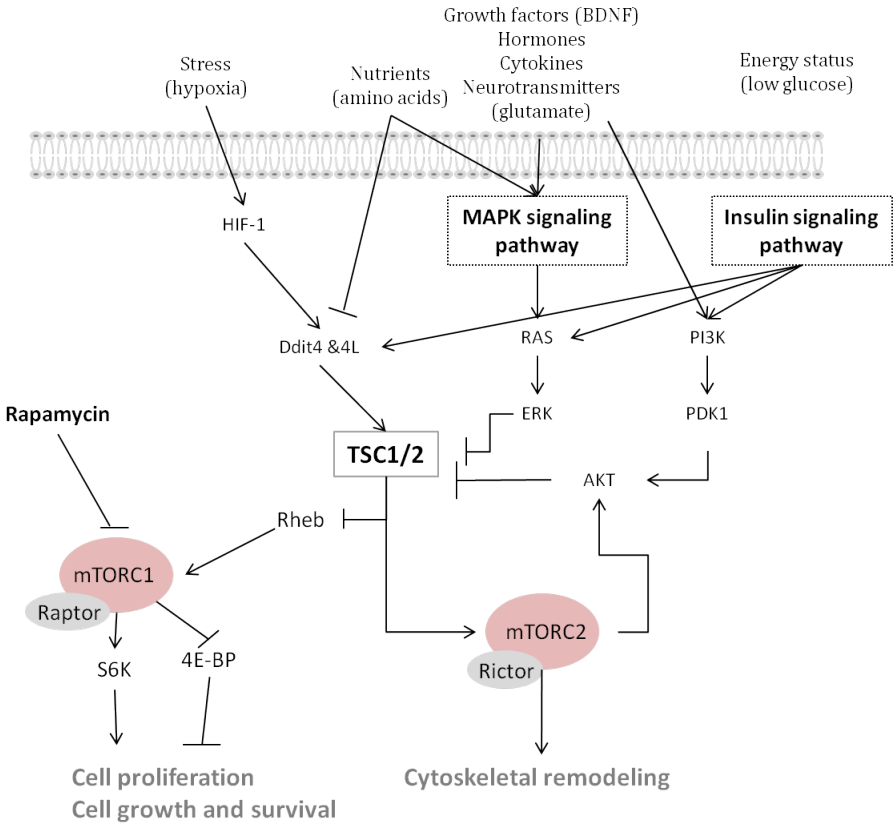

Figure 1.7: The mTOR pathway and its regulators.
Extracellular stimuli such as stress, nutrients, energy status, neurotransmitters and hormones activate intracellular pathways such as the MAPK signaling pathway and the Insulin pathway. Ultimately, this affects mTOR pathway functioning and the processes it is associated with. This includes cytoskeletal remodeling, cell proliferation and cell growth and survival.

ation (LTP) and long-term depression (LTD) (Hou and Klann, 2004; Leal et al., 2014; Ru et al., 2012). As a consequence intracellular pathways, such as the insulin signaling pathway and MAPK signaling pathway are activated, mediating the effect of the extracellular information. Hereby the expression and activity of pathway proteins are affected such as phosphoinositide dependent kinase-1 (PDK1), phosphatidylinositol 3-kinase (PI3K), v-akt thymoma viral proto-oncogene 1 (AKT) and Tuberous sclerosis protein *1* (TSC1) and *2* (TSC2) (Fang et al., 2013; Moghbelinejad et al., 2014; Komatsuzaki et al., 2012) (Figure 1.7).

The two protein complexes mTORC 1 and mTORC2 differ based on their protein-composition as well as on their substrates and their final functional implications within the neuronal cell. Whereas mTORC2 is mainly associated with cytoskeletal remodeling and regulation of cell survival, mTORC1 is involved in mRNA transcription, mRNA processing and protein translation influencing processes like cell prolif-

eration, energy regulation and cell growth and survival (Figure 1.7). The processes are affected in response to environmental/physiological changes such as hypoxia, nutrients, energy status and presence/fluctuations of growth factors, hormones and cytokines (Lipton and Sahin, 2014).

Within the CNS, the mTOR pathway has been associated with synaptic plasticity, memory retention, neuroendocrine regulation associated with food intake and modulation of neuronal repair following injury (Pilar-Cuellar et al., 2013). Malfunctioning of the mTOR pathway has been related to a number of neurological diseases such as autism and tuberous sclerosis, as well as neurodegenerative diseases as Alzheimer's, Parkinson's and Huntington's disease (Bourgeron, 2009; Chong et al., 2012; Malagelada et al., 2008; Mozaffari et al., 2009; Pei and Hugon, 2008; Williams et al., 2008) More recently, the mTOR signaling pathway has been found to be compromised in Major Depressive Disorder (MDD) (Jernigan et al., 2011) and modulation of mTOR is suggested to be a novel approach to develop strategies for the treatment of affective disorders (Dwyer et al., 2012).

The mTOR pathway and glucocorticoids

As discussed above, it is clear that both the mTOR pathway and CORT play a role in neuronal plasticity. While it is known that the mTOR pathway is subject to regulation by CORT in the periphery (Shah et al., 2000a; Wang et al., 2006a), this has been studied less well in the context of the brain. There are studies that have described an inhibitory effect of CORT on mTOR signalling in neuronal cell cultures, in rat hypothalamic organotypic cultures and mouse cortical primary cultures (Howell and Manning, 2011; Shimizu et al., 2010). More recently it has been shown that chronic stress resulted in an increase in GR and mTOR mRNA expression in the rat hippocampus (Orlovsky et al., 2014). This mRNA increase was accompanied by a significant reduction in the number of neuronal and astroglial cells. It is becoming more and more apparent that there is a connection between CORT, mTOR and neuronal plasticity, but the underlying mechanism remains to be elucidated.

In this thesis we used ChIP-Seq to investigate whether any of the mTOR proteins could be identified as primary GR-targets, which may shed light on the crosstalk between CORT, mTOR and neuronal plasticity.

1.5 Scope and outline of the thesis

In the last few years, the knowledge regarding the action of CORT within the brain has expanded enormously. Fundamental for this progress was the discovery that the central action exerted by CORT is mediated by MR and GR, which are abundantly expressed in the limbic system (hippocampus, septum, amygdala), brain regions with a crucial function in emotional expressions such as fear and aggression, learning and memory processes, and stress regulation. At the start of the research described

in this thesis, which was March 2007, the genome-wide inventory of CORT-target genes (Datson et al., 2001b) was not yet complete because of technical limitations. Due to the rapid evolution of innovative technologies, resulting in the first ChIP-seq studies, it became possible to perform genome-wide identification of transcription factor binding sites (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Reddy et al., 2009; So et al., 2007; Yu et al., 2010).

The aim of this thesis was to apply ChiP-seq combined with *in silico* methodology for identification of whole genome CORT-target genes in a neuronal setting. For this purpose we used a neuronal cell culture and also the rat hippocampus, of which we made an inventory of all genomic DNA binding sites for GR and validated several of these using RT-qPCR. In addition, we investigated the potential of MR to bind to a selection of GR-binding sites in the rat hippocampus. The thesis concludes with a study in rats aimed to investigate the genomic effect of an acute CORT challenge in the context of prior exposure to chronic restraint stress using microarray and ChIP-seq analysis. The results revealed the hippocampal mTOR pathway as a novel direct validated CORT target and showed that this pathway became particularly responsive to CORT following prior exposure to chronic stress.

Objective

The objective of this thesis is to identify all primary genomic targets for GR in a neuronal context by combining *in silico*, ChIP-seq and microarray analysis and then to apply this knowledge in a more detailed analysis of the mTOR pathway in discrete hippocampal regions: the CA3 pyramidal cell field and the dentate gyrus.

Specific aims

The common denominator in all chapters is the identification of primary genomic targets of GR within neuronal tissue. Specific aims of this thesis are:

- To use an *in silico* approach with the goal to predict neuronal-specific GREs in the genome followed by their experimental validation. For this purpose we have developed the Position Specific Scoring Matrix (PSSM) GenSig.
- To identify genome-wide GR-binding sites (GBS) *in vitro* in neuronal PC12 cells and *in vivo* in rat hippocampus using ChIP-seq and to identify genes located in the vicinity of these GBS that are activated/repressed by GR in a neuronal-specific context.
- To investigate whether MR binds to the same GBS as GR in the hippocampus and to measure binding of both receptors to these DNA sites in response to different concentrations of ligand.

- To translate the genome-wide knowledge regarding GBS into a functional application by investigating how chronic stress affects GR-mediated action of acute glucocorticoid exposure to the mTOR pathway as a novel mechanism involved in the regulation of brain plasticity.

Outline

In **Chapter 2** we show that a position-specific scoring matrix can be used to predict the presence of GREs near hippocampal target genes. Furthermore, we analyze the presence of motifs that flank the GRE-sequence which might determine cell-specific transcriptional regulation by CORT within hippocampal neurons.

In **Chapter 3** the identification of genome-wide GBS by ChIP-sequencing in neuronal PC12 cells after continuous stimulation with DEX is reported. We analyze the GBS for the presence of GREs and other known protein-binding motifs. To understand the possible functional implication of GR-binding, we identify the genes nearest to the GBS and perform gene ontology analysis. Finally we compare the GBS that are identified in this neuronal context with known GBS identified in other ChIP-seq studies.

In **Chapter 4** the analysis of genome-wide GBS using ChIP-sequencing is extended to the *in vivo* rat hippocampus after the administration of CORT. An extensive validation of a selection of GBS is presented at varying concentrations of CORT, allowing GR-GBS interaction to be studied in more detail. Furthermore, we measure concentration-dependent MR-binding to this selection of GBS to investigate whether MR and GR bind to the same genomic sequences and to get more insight into the implications of the MR/GR balance under different ligand-conditions.

In **Chapter 5**, the effect of CORT-treatment on the mTOR pathway, a major pathway in cell survival and plasticity, in discrete regions of the rat hippocampus is reported. We compare the effect of acute CORT treatment in the absence or presence of a history of chronic stress experience on the expression of mTOR pathway regulators using microarray analysis. We also investigate with ChIP-seq the capability of GR to bind near these regulators. Furthermore, we measure mTOR protein to see whether CORT and chronic stress can actually influence the mTOR pathway within the rat hippocampus.

In **Chapter 6**, the findings are evaluated and placed in context where their relevance for the stress response is discussed.