Genomic glucocorticoid signaling in the hippocampus: understanding receptor specificity and context dependency
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CHAPTER 1

General introduction

Stress – who has not experienced this in their lives, especially in this modern, fast paced world? Being stressed can be useful and even essential for survival in the case of escaping an acute physical threat. However, many people nowadays suffer from more chronic stress, which can be maladaptive and detrimental. Excessive stress can make you sick, cause psychosomatic symptoms such as headache, muscle pain or nausea, and could eventually lead to burn-out, depression or post-traumatic stress disorder (1, 2). Stress can also play a role in the development of a variety of other diseases such as epilepsy, diabetes and Alzheimer's disease (3-5). The physiological mediators of the stress response, such as stress hormones, can be used to target the stress system. There is much to gain in drug therapy of stress-related diseases with regard to specific targeting of the stress system, to reduce side-effects as well as increase effectiveness (6). Before we can better intervene in an out-of-balance stress system, it is of essence to understand in more detail how the players act in the healthy state. The different molecular aspects of the stress system are described below, leading to the goal and outline of this thesis.

Stress, neurotransmitters and hormones

In the stress system two endocrine signaling pathways are of relevance: the fast-acting catecholamines (e.g. adrenaline) and the slow-acting corticosteroids (7). Stress, a response to perceived physical or psychological threats, rapidly activates the sympathetic nervous system which leads to a fast increase in adrenaline release from the adrenal glands. Within the brain, the catecholamine neurotransmitter noradrenaline becomes more active. In parallel, the hypothalamus-pituitary-adrenal (HPA) axis is activated (**Figure 1**). Hypothalamic cells communicate via corticotropin-releasing hormone (CRH) with the pituitary gland, which in turn releases adrenocorticotropic hormone (ACTH). The adrenal glands respond to ACTH by producing the corticosteroid hormones (cortisol in humans, corticosterone in rodents). Hormones coordinate the activity of a diversity of organs and cells in the context of specific challenges to the organism. Their actions include effects in the brain, and in addition to negative feedback on the HPA axis itself, strengthening of the formation of stressful memories is a prime example (8). In fact, the two described pathways interact in this process, and especially the hippocampus has been demonstrated to be sensitive to both noradrenaline and corticosteroids (9, 10). Although there are effects in many brain areas of the memory network, like the amygdala and prefrontal cortex, we here concentrated exclusively on effects of stress hormones in the hippocampus.

This thesis focuses on the role of corticosterone, the corticosteroid that is produced by the adrenal cortex of (laboratory) rats and mice. We assume that all findings also apply to cortisol, the dominant stress steroid in humans, even if some differences exist between the two (11). Corticosteroids signal via binding to receptors that act in large measure as transcription factors. Two receptor types mediate the effects of corticosterone: the glucocorticoid receptor (GR), coded by the *Nr3c1* gene, and the mineralocorticoid receptor (MR), coded by *Nr3c2*. MRs and GRs differ in structure, affinity for different ligands, tissue expression, crosstalk partners, and as a consequence serve different roles as mediators of the many corticosterone effects on the brain. While MR is involved in the initial stress response and its gain of function variant protects against depression (12), the GR promotes stress recovery, but its chronic activation can lead to stress-related diseases such as depression (13). A major part of this thesis is dedicated to understanding how MR and GR mediate different effects of corticosterone on gene expression in the hippocampus.
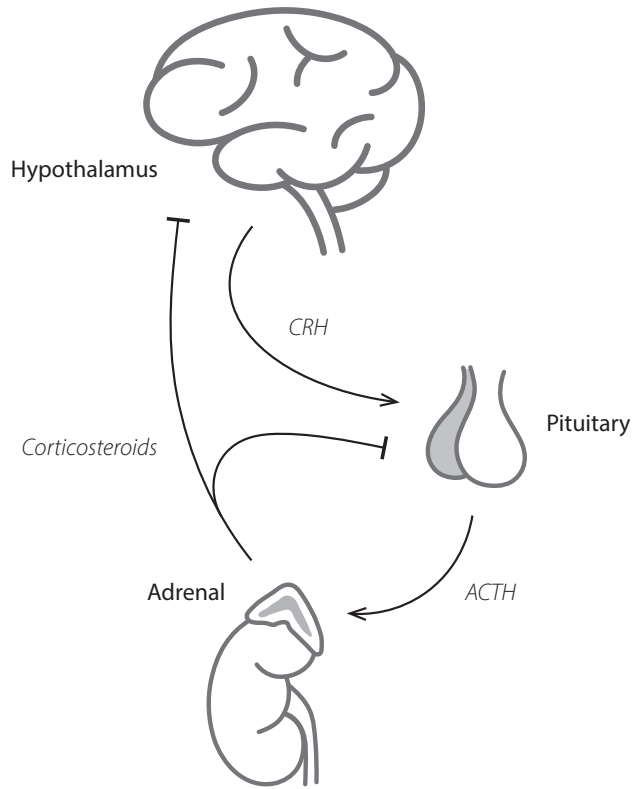
Figure 1. Graphical representation of the Hypothalamus-Pituitary-Adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) from the hypothalamus can stimulate adrenocorticotropic hormone (ACTH) release from the anterior pituitary, in response of which the adrenals produce corticosteroids. Negative feedback takes place at the level of the hypothalamus and pituitary.

The 'orchestrating' nature of corticosteroids is translated to coordinated changes in the transcription of hundreds or thousands of genes upon exposure to the hormone. These effects have a stunning dependence on cell type and cellular history: a three week period of stress leads to 50% of the corticosterone target genes to become unresponsive, while the same number of previously unresponsive genes becomes reactive to the hormone (14). Several mechanisms may explain such changes in responsiveness. In one of the chapters of this thesis we will experimentally address the crosstalk between corticosteroids and noradrenergic signaling. This introduction will describe the current knowns and unknowns of the ways in which cell- and context-specific corticosteroid transcriptional actions can take place.

Pharmacology and expression of the corticosteroid receptors

Pharmacology

Cytosolic and cellular binding assays show that MRs have a 10-fold higher binding-affinity of cortisol/corticosterone, compared to GRs. The latter are therefore better sensors for elevated hormone levels as they occur during the peak of the circadian rhythm and after stress. This difference in affinity has led to the notion that MR-dependent effects set initial reactivity to stressors, while the lower affinity GR is responsible for the response to stressors, be it dampening or sustaining (1). Of note, rapid non-genomic effects mediated by membrane-associated fractions of MRs and GRs need much higher concentrations of hormones to be activated (15). Apart from immediate effects on cellular excitability (7), these rapid effects may also set the context in which the classical MR/GR-mediated effects on gene transcription take place.

Localization and regulation

One good reason for the existence of multiple receptors for any hormone is that tissues need to respond differentially to conditions associated with increased hormone concentrations. Accordingly, MR and GR differ in their localization in brain. The classical picture from rodent brains shows that almost all brain nuclei express the GR (10), with the notable exception of the suprachiasmatic nuclei (16). The hippocampal CA3 region has substantially fewer GRs than other parts of the hippocampus. MRs have a more restricted expression pattern and are abundant in the hippocampus but also important for other limbic brain structures, such as amygdala and prefrontal cortex.

In most brain areas MRs act as receptors for corticosteroids. Co-expression with the enzyme 11-β hydroxysteroid-dehydrogenase type 2 (11β-HSD2) leads to inactivation of corticosterone, rendering MRs accessible to the mineralocorticoid hormone aldosterone. Within the brain, the nucleus of the solitary tract seems to be the main nucleus in which aldosterone-sensitive MRs reside (17, 18). On the other hand, high levels of hippocampal 11β-HSD *type 1* (11β-HSD1) drive the regeneration of active hormone from inactive metabolites, resulting in locally increased corticosterone levels (19).

The genes coding for MR and GR both have alternative promoters that are associated with splice variants that differ in their first exon, but code for identical proteins (20-22). This promoter diversity leads to differential sensitivity of the receptor genes for

regulatory factors and regulation expression, even if only a limited number of promoters dominate the expression of GR (23). Regulation of the receptor levels has received much attention, in particular with respect to early life programming of GR levels in relation to vulnerability/resilience to psychopathology, but is outside the scope of this thesis.

In the brain MRs are almost always co-localized with GRs, but many cells only express GRs. Given the higher affinity of MRs, co-localization leads to differential sensitivity for corticosterone. This may result in either linear (in case of similar effects) or bell-shaped (in case of opposite effects) dose-response curves for corticosterone. No matter what the exact relationship is, the presence of the two types of receptors ensures a broad range of responsiveness to circulating hormones, and gives the potential for a differential response to demands imposed by circadian time or stress (**Figure 2D**). However, the relationships between the effects at the molecular level and those at the (cellular or behavioral) functional level have remained for most part unresolved.

Structure of the receptors

MRs and GRs belong to the superfamily of nuclear receptors (NRs), and share the more or less modular structure that is characteristic for this class. The central DNA binding domain (DBD) is highly similar for MRs and GRs, leading to indistinguishable binding to DNA in *in vitro* settings (24). Yet, *in vivo* MR and GR activation can have opposite effects even within one cell type, and preferred or even selective target genes (25). This suggests selective DNA binding mechanisms *in vivo*, which depend on contributions of other domains of the proteins. In fact, also androgen and progesterone receptor DBDs share very high homology with that of MRs and GRs (26). The extent to which binding to common elements occurs in *in vivo* settings - forming a substrate for functional crosstalk between sex and stress steroids - has remained largely unexplored to date (27).

The C-terminal part of the receptors forms the ligand binding domain (LBD) that harbors the ligand binding pocket and an output domain referred to as Activation Function 2 (AF-2). The LBD shares substantial homology between MRs and GRs, resulting in overlapping but distinct pharmacology and shared downstream AF-2 signaling partners. The GRβ is a splice variant of the receptor that lacks part of the LBD. This splice variant may be of relevance to glucocorticoid signaling in the immune system, but under control conditions its levels in the brain are negligible (28, 29). LBD-lacking MR splice variants have also been characterized, but their relevance for brain function remains unknown (30).
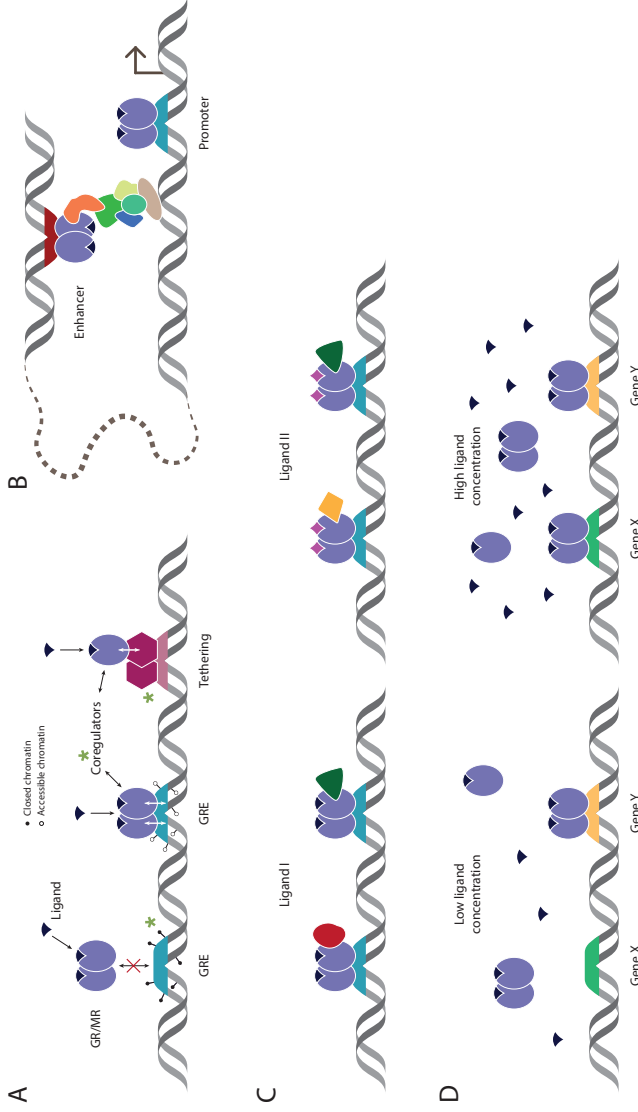
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Figure 2. Basic principles of MR/GR transcriptional regulation. **A)** Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) binding to DNA is dependent on several interactions: ligand binding can alter the receptor conformation; a priori chromatin accessibility can direct receptor binding; binding of MR/GR to the DNA is a two-way interaction in which the receptor and the DNA influence each other's conformation; and lastly receptors cooperate with coregulators that modulate the transcriptional effects. Besides direct DNA binding through the glucocorticoid response element (GRE), GR and MR can also tether to other proteins such as AP-1 and NF-κB, to bind the genome indirectly. *Chromatin landscape, coregulators and tethering proteins can be cell- and tissue-specific and contribute to the diversity in MR/GR transcriptional effects. **B)** MR/GR not only bind to promoter regions to function as transcription factors, but are also found at distal sites from transcription start sites. At these sites MR/GR function as enhancers, and DNA loops in order to interact with the transcriptional machinery of the modulated gene. **C)** Different ligands can lead to recruitment of different coregulators. This principle can be exploited for the development of selective receptor modulators – which allow some interactions, but prevent binding of other coregulators – to selectively target MR/GR downstream pathways. **D)** Transcriptional effects can be dependent on the ligand dose, with sensitive genes already being regulated at low ligand concentrations (Gene Y), while other genes might get bound and affected only at higher ligand concentrations (Gene X). This can depend e.g. on the MR/GR difference in ligand affinity, but also on the GRE sequence, presence of surrounding transcription factor binding sites, chromatin modifications, and gives cells the opportunity to selectively modulate a subset of target genes depending on the hormone level and associated body's demand.

The N-terminal domains (NTDs) of the receptors are much less well understood, due to their so-called intrinsically unstructured nature (31). MR and GR NTDs contain an Activation Function-1 (AF-1) that interacts with sets of downstream proteins, by which part of the actual signaling occurs. NTDs are highly specific to the receptor type, and ever since cloning have been considered the basis for differential effects that are mediated by MRs and GRs. Of note, due to alternative translation start sites on the GR mRNA, several translation variants exist (GR-A, GR-B, etc.). These different N-terminal truncations have tissue-specific expression, and are known to differ functionally (32, 33). Yet, for lack of specific tools to determine these variants, we have little understanding of the role of translation variants in physiological (brain-related) processes.

Numerous amino acids in the MR and GR proteins are subject to posttranslational modification, including phosphorylation, SUMOylation and acetylation (34). The consequences range from changes in ligand binding and the subsequent nuclear translocation to target gene identification (35) and transcriptional activity (36, 37). Most of the specific consequences of diverse modifications have been identified for the GR, mainly within cell lines representing peripheral tissues. Thus, specific relevance for brain function remains often unclear. However, recent data have shown that BDNF signaling impacts GR transcriptional activity via phosphorylation of the receptor in primary cortical neurons and this likely occurs *in vivo* within the hypothalamus as well (38, 39). Clearly, the range of posttranslational modification is a basis for extensive crosstalk with other signaling pathways, and a structural basis for the context-dependence of MR- and GR-mediated signaling. This is complemented by regulation of GR expression by classical transcription factors (40) and microRNAs (41).

Signaling modes of the receptors

The genomic modes of MR and GR action can be divided into two types (**Figure 2A**). The first is the classical action of direct binding to the DNA, i.e. binding sites that harbor a glucocorticoid response element (GRE). This consensus sequence is a palindromic sequence (AGAACANNNTGTTCT, or many variations thereof (42)) which enables binding of receptor dimers, with each of the subunits interacting with one of the GRE half sites, separated by a 3-bp spacer. Classical GR targets often used to probe GR responsiveness of tissues are *Per1*, *Tsc22d3* (encoding GILZ) and *Fkbp5*. Also many classical liver target genes coding for catabolic enzymes are GRE dependent (43). Transcriptional output is then mediated by recruitment of downstream coregulator proteins that recruit RNA

polymerase II to the promoter, or facilitate transcription in other ways (44). On the other hand, the receptors can alter gene transcription via 'tethering' mechanisms at non-GRE binding sites for other transcription factors. At these sites MR/GR monomers use protein-protein interactions to bind other transcription factors, preventing their transcriptional effects. Examples of transrepressed proteins are AP-1 and NF-κB (45). Of note, in rats that were injected with corticosterone under resting conditions, GR was found almost exclusively at GRE-containing loci (46). In the GR$^{dim/dim}$ mouse, that carries GRs that are selectively impaired in GRE binding, there are clear disturbances in GR-mediated effects on hippocampal function, indicative of GRE-dependent processes. Pituitary ACTH is very high in these mice, but plasma ACTH levels are close to normal, pointing to either protein-protein interactions in the regulation of ACTH release (47-49), or the incomplete abrogation of the DNA binding capacity of these mutated GRs (50).

Although direct DNA binding is often associated with transactivation and the tethering mechanism with transrepression (in anti-inflammatory contexts), this dichotomy is not that clear-cut. For example, there are negative GREs (nGREs), at which GR binding induces downregulation of the target genes. The *Crh* promoter harbors such a nGRE that enables dexamethasone-induced lowering of its gene expression (51). Similar nGRE-containing sites have been demonstrated in the promoter regions of *Pomc* (52) and the inflammation gene *IL-1β* (53). At these sites the GR can transrepress via direct DNA binding. The nGRE differs from the classical GRE in structure and functionality, as the inverted repeat allows a variable spacer length (54) and GR can bind these sites in a monomeric manner (55). In fact, it has been claimed that many NF-κB sites contain nGRE-like sequences that are recognized by GR monomers (56). In the same line, GR has been shown to bind a subset of AP-1 sites without activation of AP-1 as a tethering factor, through direct binding of an embedded GRE half site (57). Interestingly, the *Nr3c1* gene coding for the GR contains a nGRE that allows homologous downregulation of GR in many cellular contexts (58). In other cases, nGREs involve 'composite' DNA-binding of GR *and* interactions with other transcription factors that bind DNA (59). Thus, direct binding to DNA can clearly lead to 'transrepression' as defined by a suppression of ongoing gene transcription. Moreover, protein-protein interactions like the indirect binding of GR via STAT5 can also lead to gene induction (60).

Understanding the relative importance of different modes of transcriptional regulation has been advanced by genome-wide identification of (in particular: GR) binding sites in different cells, organs and contexts. The method of choice for this is chromatin-immunoprecipitation (ChIP) followed by sequencing (ChIP-seq), and advanced variants

of this approach such as ChIP-exo and ChIP-nexus (61-63). Amongst the crucial steps in a ChIP assay are DNA-protein crosslinking, chromatin fragmentation and the immunoprecipitation, for which antibodies need to be carefully considered (64, 65). After purification of the pulled-down DNA fragments, sequencing is used as a readout for whole genome binding, whereas qPCR can be performed downstream (ChIP-qPCR) in case target loci are known (**Figure 3**). Processing of ChIP-seq data involves read alignment, in combination with quality control filters, peak calling and may include follow-up analysis such as differential binding, peak annotation (for genomic distribution and gene ontology) and motif discovery. Several similar bioinformatics tools exist for each of the data analysis steps. Methods may be dependent on the platform used for ChIP-seq, the type of factor studied (e.g. histone mark, polymerase or transcription factor), and research groups can have their own preferences and algorithms (64, 66). Most of the genome-wide GR binding data derive from ChIP experiments in non-neuronal cell cultures (35, 67-70). Though *in vivo* (or *ex vivo*) whole genome GR binding has been examined with this technique in various tissues (71-73), relatively few ChIP-seq datasets are available for GR in the brain (46, 74). Studies on the MR cistrome in cell lines are scarce and limited to aldosterone-stimulated conditions (75) or transiently expressed receptors (63), let alone that binding data has been collected for this endogenous receptor in any tissue. In this thesis we concomitantly study rat hippocampal MR and GR for their genome-wide binding landscapes.

Besides regulation of mRNA transcription, MR and GR can also exert their effects on the genome via indirect mechanisms such as controlling non-coding RNAs. Glucocorticoids can increase the expression levels of miRNA-27b in adipose tissue, thereby blocking a crucial differentiation gene and preventing browning of the white fat cells (76). Another example is the induction of miRNA-511 by GR signaling that can protect against TNF-induced shock (77). Also retrotransposons have been found to be regulated by glucocorticoids. Acute restraint stress can increase the H3K9 trimethylation of these transposable elements in the CA3/DG region of the hippocampus, thereby preventing their expression (78). The role of retrotransposons in the light of stress and adaptation has been extensively reviewed by the Hunter laboratory (79, 80). Conceptually, the functional consequences of regulation of these non-coding RNAs can be considered different from classical target genes. For example miRNAs by themselves have a wide range of translational targets, and therefore their regulation via GR (or MR) entails a distinct form of 'coordinating coordinators'. Alternatively, miRNAs that are regulated via MR or GR may be seen as large-scale amplifiers, or second order mediators of the initial steroid effect.
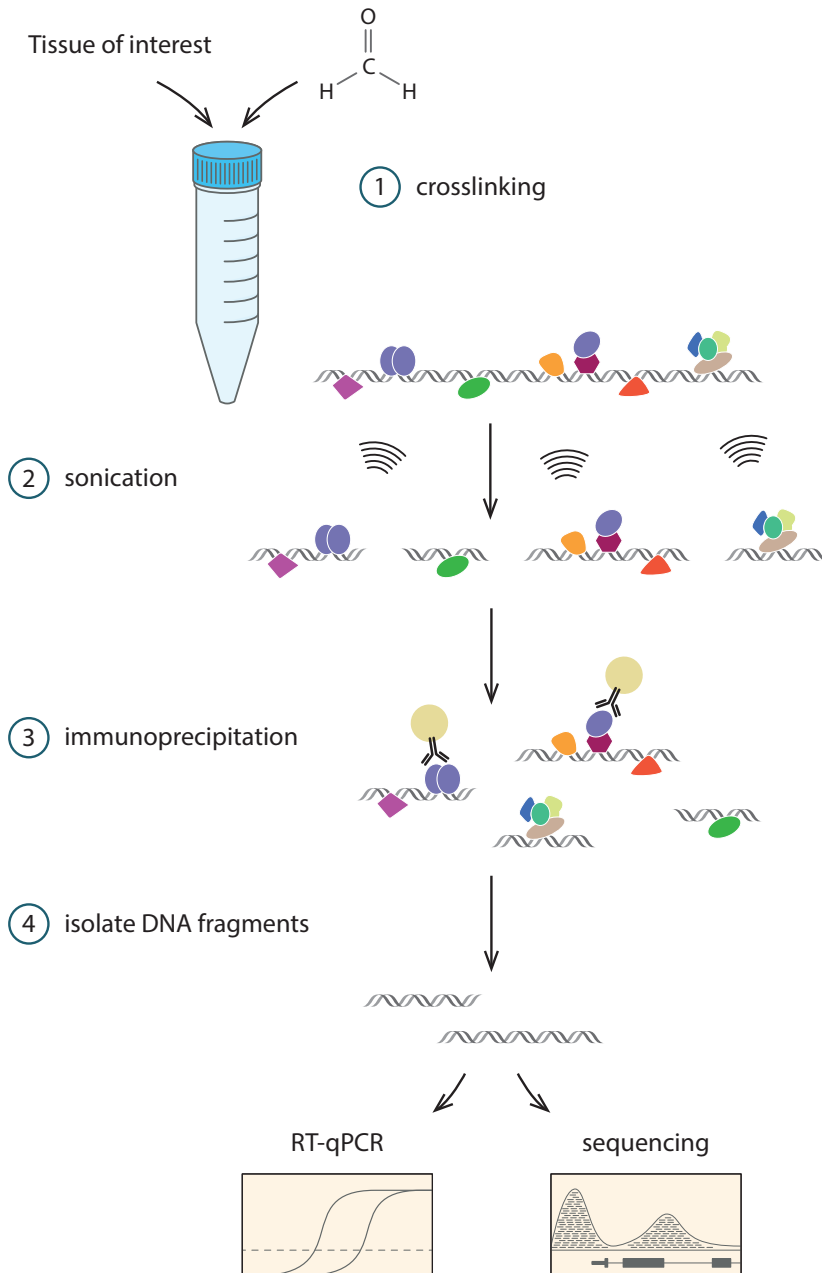
Figure 3. Illustration of the different steps during a chromatin-immunoprecipitation (ChIP) experiment. After **[1]** DNA-protein crosslinking using formaldehyde, **[2]** chromatin fragmentation by means of sonication and **[3]** immunoprecipitation with antibodies directed against the protein of interest, **[4]** purified pulled-down DNA fragments can be analyzed either at the individual level by real-time quantitative PCR (RT-qPCR) or at the whole genome level by sequencing.

Interaction with DNA

The direct binding of MR/GR to the DNA is a two-way interaction between the DBD of the receptor and the DNA sequence (**Figure 2A**). Ligand binding directs the receptor into a particular conformation, which favors interactions with GREs. In turn, the DNA sequence of the binding site also promotes conformational changes and alters the exact structural ordering of AF-1 and AF-2 domains. Consequently, the exact GRE sequence determines not only the DNA-binding affinity of the receptors but also their activity, in conjunction with the conformation induced by ligand binding (81). Also receptor dimerization has consequences for sequence-specific conformational changes (82). A variation on this theme is the relevance of the number of GREs that are in close vicinity to each other. This was brought to light by the unexpected finding that mutations that render the GR ineffective in binding to single GREs (49) in fact are very potent binders to multiple GREs that can be present in natural promoters (50). Accordingly, the involvement and efficacy of downstream coregulators differs as a function of GRE sequence and number (83). Concerted action on multiple GR binding sites might even be required for successful transcriptional regulation (84) and can explain why isolated binding events do not necessarily warrant gene expression changes.

Transcription factors are often viewed as promoter binding proteins. However, MR and GR also bind relatively distant from genes **(Figure 2B)**. Almost half of the GR binding sites within the rat hippocampus are located more than 10 kb from a gene (46). In a human kidney cell line 84% of the MR occupied sites are over 10 kb from a transcription start site (75). At the more distant binding sites, MR and GR can function as enhancers, inducing the folding and looping of DNA to influence promoter regions (69, 84). There are also differences in proximity and kinetics of binding between activated and repressed genes (85). DNA sequences are frequently depicted as linear structures, but clearly have a complex 3D structure that extends beyond local chromatin structure.

Much of the cell-specificity depends on the local chromatin status of the genomic binding sites (**Figure 2A**). GR binds mainly in DNA regions that are accessible before hormonal activation (42). A small subset of binding sites shows open chromatin only after glucocorticoid treatment, suggesting that GR can also serve as a pioneering factor to attract chromatin remodelers and induce long-lasting changes in gene accessibility. Moreover, effects of long-range GR interactions also vary depending on preexisting DNA accessibility (69). Several factors can define the GR cistrome. E.g. AP-1 maintains an open chromatin structure favoring GR binding (86) and also coregulator Hic-5 can assist in binding site selection (87). On top of that, even the same GR binding site has been shown

to cell type-dependently interact with distinct alternative promoters of the same gene (*Tsc22d3/Gilz*) as a result of cell type-specific 3D chromatin organization (84).

Interactions at the GRE with nearby transcription factors

The presence of a GRE is not necessarily linked to MR/GR binding, but the conservation of a particular GRE is predictive for its functionality (88). In fact, there are many more GRE sequences in the genome than actual binding sites for MR or GR in a given cell type. Profiling of binding sites in two unrelated cell types resulted in thousands of binding sites that showed less than 10% overlap between the two (42). The extent of overlap in binding sites between different brain regions or neuronal cell types may be higher, but is currently unknown. Genome-wide studies have revealed that functional GR binding sequences (GBS) in particular tissues often co-occurs with binding motifs for other transcription factors (89). These enriched binding sites may represent binding of cell lineage-determining factors, and cell type-specific 'hotspots' of transcription factor binding. Alternatively, they may point to receptor-specific signaling partners that are involved in creating the impressive cell-specificity of steroid receptor mediated transcriptional regulation.

The potential synergy between transcription factors at a single genomic locus was emphasized in an elegant study in cell lines addressing the interaction between GR and an estrogen receptor (ER) that contained the GR DBD, instead of its own. Rather than competition for the same binding site, the authors observed that these two transcription factors cooperated at a common binding locus (90). Thus, residence time at the DNA is short enough to enable joint action, and the different output functions of transcription factors allow for substantial synergy. This is of interest, as MR and GR share the same GRE (75). First, these receptors may heterodimerize (91, 92), but an alternative option is that they co-bind to the same site as homodimers in a dynamic manner (residence time depending on the particular ligand). Besides the classical view of dimer (and monomer) binding, also GR tetramers have been reported (93). This higher order oligomeric state seems to be triggered upon DNA binding and tetramers could occupy a single locus, but might also be of relevance in looping events. In addition, more complex modes of interaction could play a role, as recently MR DBD mutants have been shown to indirectly bind glucocorticoid target loci via tethering to GR (63).

The overlap in binding sites of steroid receptors may also be relevant for the interaction between sex steroids and stress steroids. At the functional level, androgen receptor

(AR) antagonism interferes with liver GR signaling, which in contrast to observations in adipocytes could not be explained by attenuated 11β-HSD1 levels and related local corticosterone concentrations (94). The GR-induced activation of *Fkbp5* and *Tsc22d3/ Gilz* that is diminished upon blockage of AR, suggests possible AR-GR interactions at the genome. This is supported by substantial overlap between the AR and GR cistrome in prostate cancer cells found previously (95), and the capability of the two receptors to form heterodimers (26). Besides interactions with MR and AR, the GR can also exhibit crosstalk with the related progesterone receptor (PR). In breast cancer cells GR inhibits PR-dependent cell proliferation, and the genome-wide binding sites of GR were shown to be highly overlapping with those of PR (96). Using sequential ChIP, the two receptors were confirmed to co-bind several shared regulatory regions. A thorough review by Ruiz et al. (97) discusses the interactions of glucocorticoids with sex-steroids, via GR with AR and PR - all of which are expressed within the brain (and also the hippocampus specifically) (98). Since the DBD of the ER is distinct from the other members of the NR family and binds independent sequences/response elements (99), the many ER-GR interactions (97) are not mediated at the level of shared *direct* binding of target loci. Nevertheless a process of assisted loading, dependent on AP-1, has been described when ER and GR were activated simultaneously (100). Furthermore transcriptional repression is demonstrated by GR tethering to ER enhancer complexes (101). In this thesis we focus on the genomic actions and interplay of the two glucocorticoid-responsive receptors.

Understanding the molecular intricacies of MR/GR interactions in different brain structures will remain a challenge, given that the preferred molecular signaling partners of the receptors seem to be highly cell type-specific. In addition, while treatment of animals at rest revealed that the majority of GR binding sites was classical GRE-dependent, the presence of interacting transcription factors would be predicted to change in situations where neuronal circuitry was highly activated (102). Besides interactions with other NRs as described above, the GR might thus have crosstalk with other transcription factors for such context-specific transcriptional regulation. In one chapter of this thesis we explore the interaction of GR with a downstream mediator of the noradrenaline pathway.

Coregulator diversity

If two perquisites of ligand binding and subsequent localization at the DNA have been met, the receptors can have their actual effects: modulation of gene expression. In direct DNA binding mode this occurs via their AF-1 and AF-2 output domains, and recruitment of downstream mediators known as NR coregulators (**Figure 2A**). These coregulators

can direct GR target gene expression by chromatin modification and recruitment and stabilization of the transcription factor complex (103). Often coactivators associate with agonist-bound receptors, while corepressors may bind to antagonist-bound receptors. Hundreds of coregulators have been described, and many of these can interact with several transcription factors. An emerging notion is that these proteins form actual integrators of the signals of individual transcription factors, that act as hubs in information processing at the chromatin (44). Of note, these coregulators are expressed and regulated in a tissue- and cell type-specific manner. As more often, even if general knowledge is available, there is sparse knowledge on specific neuronal circuits.

Activation Function 1

The N-terminal domain (NTD) of the MR/GR comprises the AF-1. Since the NTD is intrinsically unstructured and interactions with this domain are ligand independent, this part of the receptor is not well studied. However, this domain is most divergent between MRs and GRs, and may be responsible for differential effects of the receptors on gene expression. Recent experiments have identified proteins that interact with full length MRs. This dataset may well contain a number of AF-1 coregulators that are unique to MR, and even may respond in a ligand-dependent manner (104). Early studies with chimeric receptors revealed that the MR-NTD is less potent that the GR-NTD in (crude) luciferase assays to probe transcriptional activity. This may reflect either intrinsic characteristics of the AF-1, or differences in post-translational modifications that take place at the respective NTDs. It has been shown that SUMO-ylation can restrict transcriptional activity, and that the MR-NTD can be more heavily SUMO-ylated than the GR-NTD (105). All in all, there is still much to learn about this part of MR/GR signaling. The relevance of understanding details of MR AF-1 is illustrated with a haplotype of the human MR gene that confers resilience to psychopathology contains a variation the NTD (besides a variation in around the mRNA translation start site) (12, 106).

Activation Function 2

The other domain that interacts with coregulators and the basic transcriptional machinery is the AF-2 in the C-terminal part of MR/GR. Binding takes place via the coregulator's NR binding domains, the so-called NR boxes containing an LxxLL amino acid signature (107). This AF-2 is dependent on conformational changes of the receptor after ligand binding, and structurally well-understood (108). Of note, based on the modular nature of the interactions, these can be well studied in an *in vitro* assay that uses the binding motifs

of coregulators on a chip, which is incubated with the LBD of any NR, to explore NR-coregulator interactions (109). This assay may be used to probe activity from endogenous full length GR from cellular context (110). This comprehensive approach for AF-2 has confirmed the substantial overlap in signaling partners of different NRs, suggesting that these AF-2 coregulators are indeed a basis for functional crosstalk between MR/GR and for example sex steroid receptors.

A number of studies have addressed the specific contribution of coregulators to MG/GR signaling in specific brain regions and processes. Absence of the members of the p160 Steroid Receptor Coactivator (SRC) family leads to various neurobehavioral consequences (111). The *Ncoa1* gene coding for SRC-1 has been studied in much more detail with respect to GR effects on the brain. Absence of SRC-1 leads to apparently full GR resistance for negative regulation of pituitary *Pomc* and brain *Crh* (112, 113). Strikingly, both hypothalamic *down*regulation and amygdala *up*regulation after glucocorticoid treatment depended on SRC-1. The tissue specificity may be explained by the existence of different splice variants of the SRC-1 protein: SRC-1a and SRC-1e (114). The functional consequences of SRC-1 absence in these mice were very limited, which has been attributed to developmental compensatory mechanisms (115). Thus, while work on SRC-1 clearly shows the potential importance of regional and context-induced differences in coregulator activity, there is veritable *mer à boire* in terms of in depth understanding their roles in MR/GR function.

Selective recruitment of coregulators by ligands

There is a number of options to get insight in the coregulator dependence of particular MR/GR-mediated effects on brain function. A first one would be comparison of genome-wide binding patterns between the receptors and individual coregulators. Such an approach needs to be supported by very good prediction of the relevant coregulators in a particular system. Combining publicly available datasets to study the degree of coexpression within certain tissues and cell types can help to narrow down factors of interest (98). There may also be another option to gain more understanding of receptor-specific effects, i.e. by using so-called selective receptor modulators (SRMs) (**Figure 2C**).

SRMs are compounds that cause a conformation of the receptor that is intermediate to that induced by full and partial agonists, and antagonists (116). As a consequence, SRMs allow interaction with some, but not all receptor coregulators (117). This in turn results in tissue- or gene-specific agonism/antagonism. The best-known example of a SRM is tamoxifen, that acts as estrogen receptor antagonist in breast cancer, but as

an agonist in bone and endometrium. However, also selective modulators for GR have been identified, in part based on the MARCoNI coregulator profiling tool mentioned earlier (6, 118). Comparing the induced effects of these drugs with the coregulator-interaction that they induce, may form a (relatively) expedient way to characterize the molecular pathways involved in individual MR/GR-dependent effects of glucocorticoids. A case in point is the SGRM CORT108297, that leads to preferential recruitment of the SRC-1a protein (compared to SRC-1e). This splice variant is involved in transcriptional repression, and this is what was observed for the HPA axis after treatment with the drug (6). The therapeutic potential of such SGRMs has been demonstrated by studies using CORT118335. This compound could prevent and reverse hepatic lipid accumulation in mice receiving a high-fat diet, by stimulating GR-dependent liver efflux, while lacking agonistic effects that corticosterone has on liver uptake of fatty acids (119).

Target genes

All these differences in the nature of binding and signaling of MR/GR to the DNA lead to gene-specific efficacy of the receptors. The genes coding for the core-secretagogues of the HPA axis (*Crh, Avp* and *Pomc*) are well known targets of glucocorticoids (120). A substantial number of individual transcriptional targets in different brain areas have been identified by candidate gene approaches, even if *direct* regulation rather than second order transcriptional changes or trans-synaptic regulation remains difficult to establish based on protein or even mRNA changes. Some of these target genes are 'generic', such as *Fkbp5, Tsc22d3/Gilz,* and *Per1*, and are often used as readouts for GR-sensitivity (121). Many other target genes show strong cell type specificity (122). More comprehensive approaches like differential display, SAGE, DNA microarrays and more recently RNA-seq have given an unbiased view of the genes that are (directly or indirectly) affected via MR/GR activation (25). However, given the vast cellular diversity in the up to 900 brain areas that have been defined (123), *and* given the strong context dependence of transcriptional responses (124), we are far from a full understanding of how MR/GR affect the brain (non-genomic effects aside).

Transcriptome (and cistrome) analyses do however give insights that go beyond the individual brain area in which results were obtained. Genome-wide analyses of (non-neuronal) cell lines showed highly divergent dose-response curves for transcriptional targets of GR. Strikingly, stimulation of the circadian clock gene *Per1* requires much lower concentration of activated receptor in the nucleus than is required for most other genes (85). This makes sense, as control of circadian processes via endogenous glucocorticoids

(125) should not be dependent on high stress levels of hormone. Conversely, stress-induced changes in gene expression should often exceed normal circadian demands (126). There seem to be several levels that determine 'genomic' GR sensitivity (**Figure 2D**). Differences between low and high glucocorticoid levels were also apparent in zebrafish, where there was no overlap in GR target genes as determined by GR knockdown and by GR overstimulation (127). In rat hippocampus, there was a difference between moderate to high to very high hormone levels, with the latter apparently leading to occupation of lower affinity DNA loci by the GR (46).

Thus, the notion is that (circadian) 'maintenance' processes are regulated via both MR and high affinity binding sites of GR, whereas adaptations to progressively more severe stressors will depend on receptor-DNA interactions that have lower affinity. In this respect the affinity of receptors for the DNA is an extension of the functional relevance of binding affinity of corticosterone for MR and GR, where a similar difference of 'preparative' and 'reactive' corticosteroid effects has been noted (128).

MR/GR 'switches'

As a last layer, we will discuss duration of the MR/GR-induced effects in the brain. Duration of endogenous glucocorticoid exposure ranges from hourly ultradian pulses (129) and transient peaks from acute stressors to longer exposure as a consequence of chronically elevated levels. Likewise, duration of effects can differ. In circadian settings, they should be in a range of hours. However, some effects last very long – e.g. facilitation of memory consolidation may be necessary for long-term memories to form (48). In experimental setup, glucocorticoids can act as an actual switch that freezes neuronal circuits in a particular state (130). Also in Cushing's disease, prolonged exposure to cortisol can have effects on gray matter volume as measured 10 years later (131). Such apparently irreversible effects may be caused by permanent changes in activity of particular target genes, via epigenetic changes involving either DNA methylation or chromatin remodeling (132). In cell lines, GR can bind to previously inactive chromatin, even if these represent a minor fraction of all loci (42). GR has also been shown to directly affect DNA methylation, in the context of an intracellular negative feedback loop involving the *Fkbp5* gene (133). Outstanding questions are why some brain areas are more vulnerable to long-term changes than others, and which exact mechanisms underpin these effects.

Outline of this thesis

We have discussed how ligand binding leads to nuclear interactions of MR/GR with the DNA or other proteins, followed by recruitment of downstream signaling partners and eventually to transcriptional regulation. Many aspects around MR/GR signaling in the hippocampus are still unclear. These include interactions between non-genomic and genomic signaling of glucocorticoids, programming effects of glucocorticoids, and interactions with NRs for other steroid hormones and different transcription factors. In this thesis we focused on two aspects. The first is how MR and GR activation can have very different effects, despite the high homology of their DBD. Conceivable scenarios are that they bind distinct GREs, or that upon binding of the same GREs the receptors differentially interact with specific coactivators/corepressors. The second question we addressed is whether and how crosstalk between GR and noradrenaline signaling can take place at the genome.

The overall aim of this thesis was to gain more understanding in the receptor specificity and context dependency of corticosteroid hormone effects in the hippocampus. Objectives of the work presented were to: 1) characterize the extent of overlap versus specificity between MR and GR binding and concomitant transcriptional consequences, and 2) study GR transcriptional effects in a stressful learning context, in which GR activation acts as a 'switch' for long-term memory consolidation, and in which an interaction with the noradrenaline system is expected.

In **Chapter 2** we have answered the long-standing question of how glucocorticoids via the structurally comparable receptors MR and GR can nevertheless elicit differential transcriptional effects. To this end we aligned the genome-wide binding profiles of MR and GR in the *in vivo* context of the rat hippocampus. We describe the overlap and differences in target location, functional annotation and peak sequence characteristics. A second type of transcription factors, NeuroD factors, was found to bind specifically near MR-bound loci. This suggests a role for these types of transcription factors driving specificity in corticosteroid receptor DNA binding and subsequent gene regulation.

In **Chapter 3** we addressed functional effects of the previously found hippocampal MR/GR binding profiles, by examining gene expression levels related to the different subgroups of MR-specific, MR-GR overlapping and GR-specific target loci. Transcriptional effects were evaluated in MR knockout animals and in an acute stress model of restraint stress. This led to the identification of *Jun dimerization protein 2* (*Jdp2*) as, at least for the hippocampus, a stress-responsive MR-specific target gene.

In **Chapter 4** we examined the mechanism by which NeuroD factors were able to direct specificity of MR over GR binding, and how they can enhance glucocorticoid transcriptional effects. We also studied whether MR binding to the DNA is necessary for binding of its partners, NeuroD and GR. Functional comparison with several NeuroD-related factors in reporter assays pointed to the conclusion that chromatin remodeling seems the main aspect underlying NeuroD-potentiated MR signaling.

In **Chapter 5** we studied the role of GR in a learning context. To this end we employed the object location memory (OLM) task, in which glucocorticoid potentiating effects are dependent on training-induced noradrenaline signaling. We hypothesized that at the level of hippocampal DNA binding there would be an interaction between the phosphorylated transcription factor cAMP response element-binding protein (pCREB), as being activated by noradrenaline, and GR in the arousing learning condition. Analysis focused on the GR dataset, for which the subset of targets was partially affected by OLM training and confirms context specificity of corticosterone-induced transcriptional regulation. Two novel hippocampal GR targets were identified, *Gap junction protein, beta 6* (*Gjb6*) and *NMDA receptor synaptonuclear signaling and neuronal migration factor* (*Nsmf*).

In **Chapter 6** the findings and implications of these studies are discussed.

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