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

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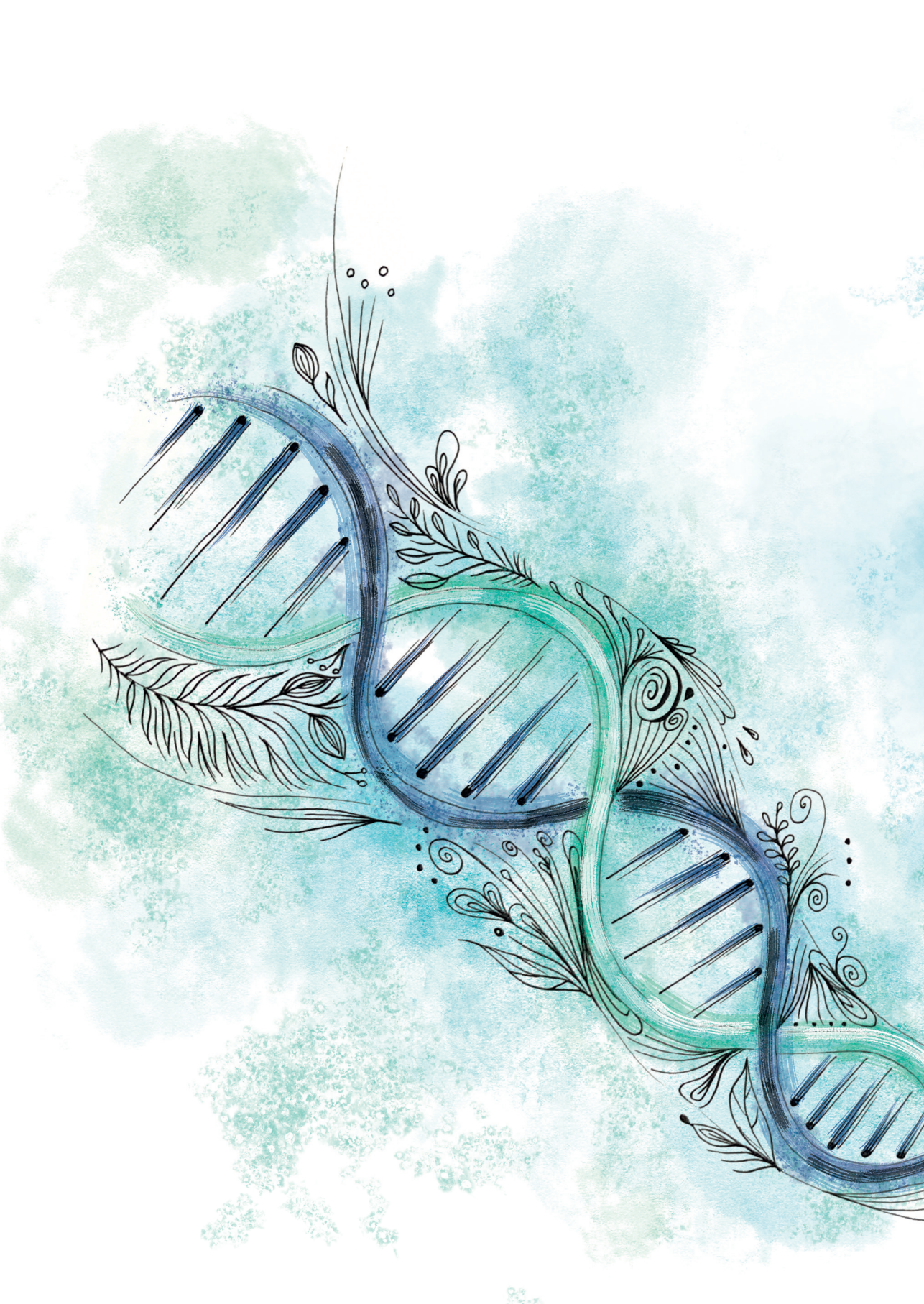

CHAPTER 1

General introduction

Adapted from:

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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.

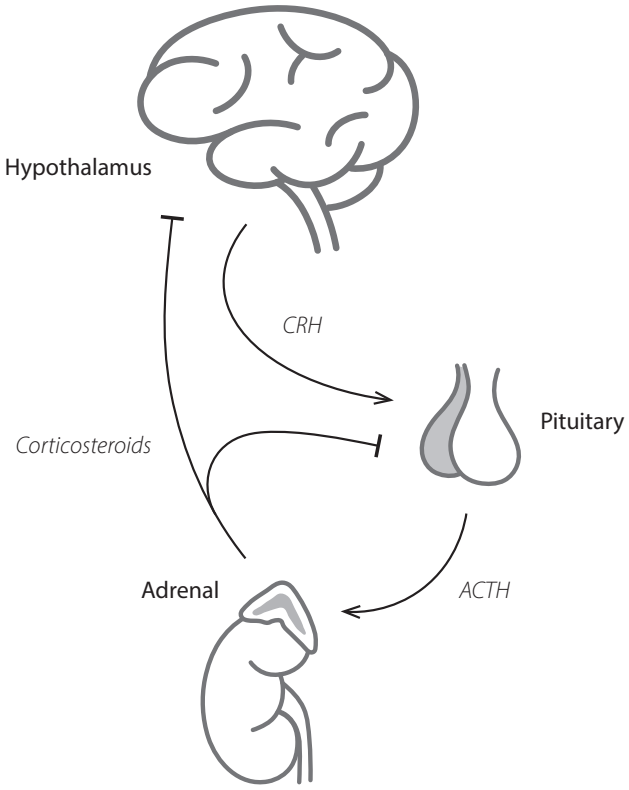

Figure 1. Graphical representation of the Hypothalamus-Pituitary-Adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) from the hypothalamus can stimulate adrenocorticotropic hormone (ACTH) release from the anterior pituitary, in response of which the adrenals produce corticosteroids. Negative feedback 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).

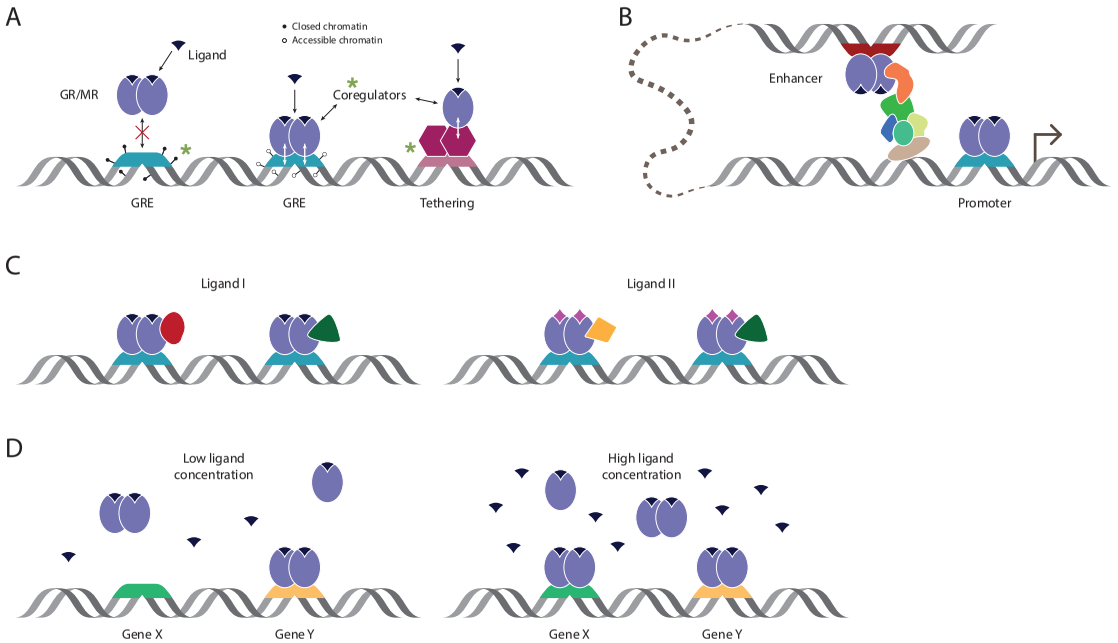

Figure 2. Basic principles of MR/GR transcriptional regulation. **A)** Mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) binding to DNA is dependent on several interactions: ligand binding can alter the receptor conformation; a priori chromatin accessibility can direct receptor binding; 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.

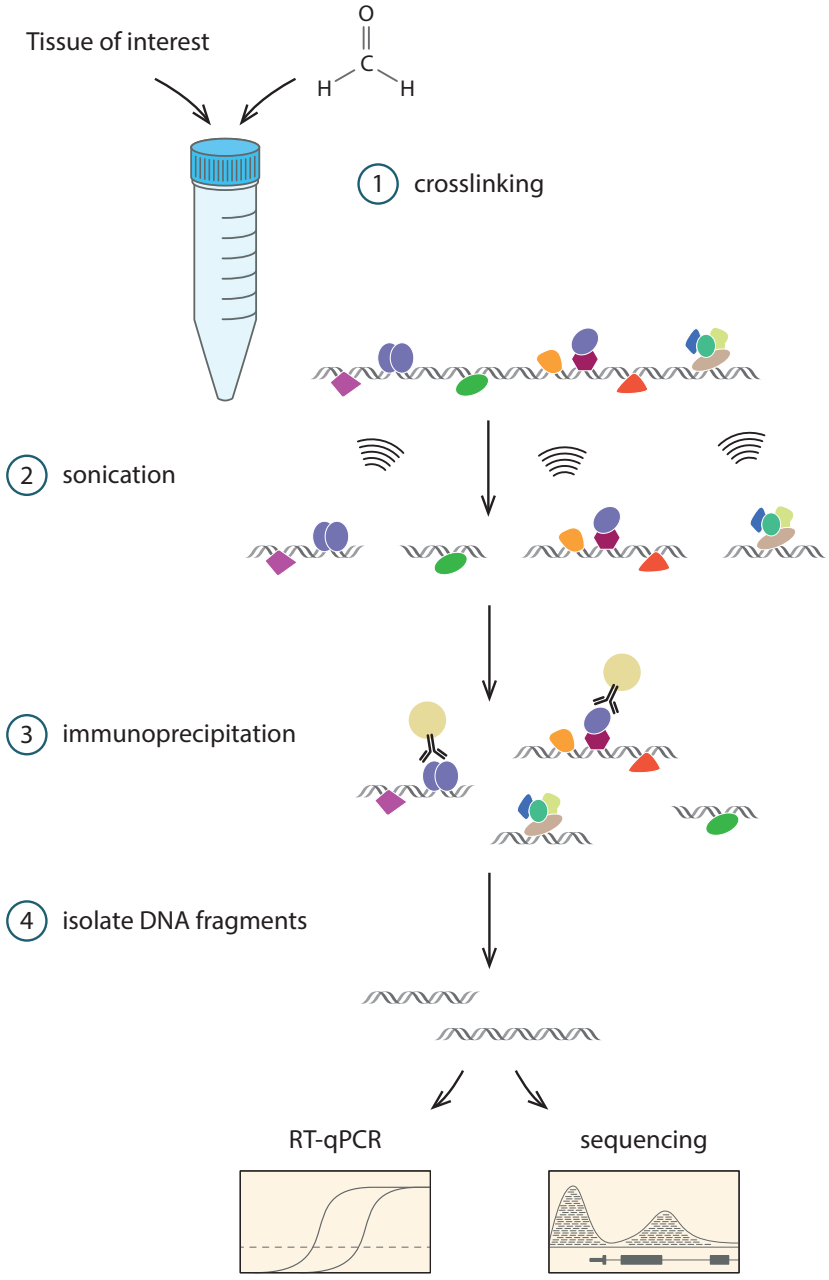

Figure 3. Illustration of the different steps during a chromatin-immunoprecipitation (ChIP) experiment. After **[1]** DNA-protein crosslinking using formaldehyde, **[2]** chromatin fragmentation by means of sonication and **[3]** immunoprecipitation with antibodies directed against the protein of interest, **[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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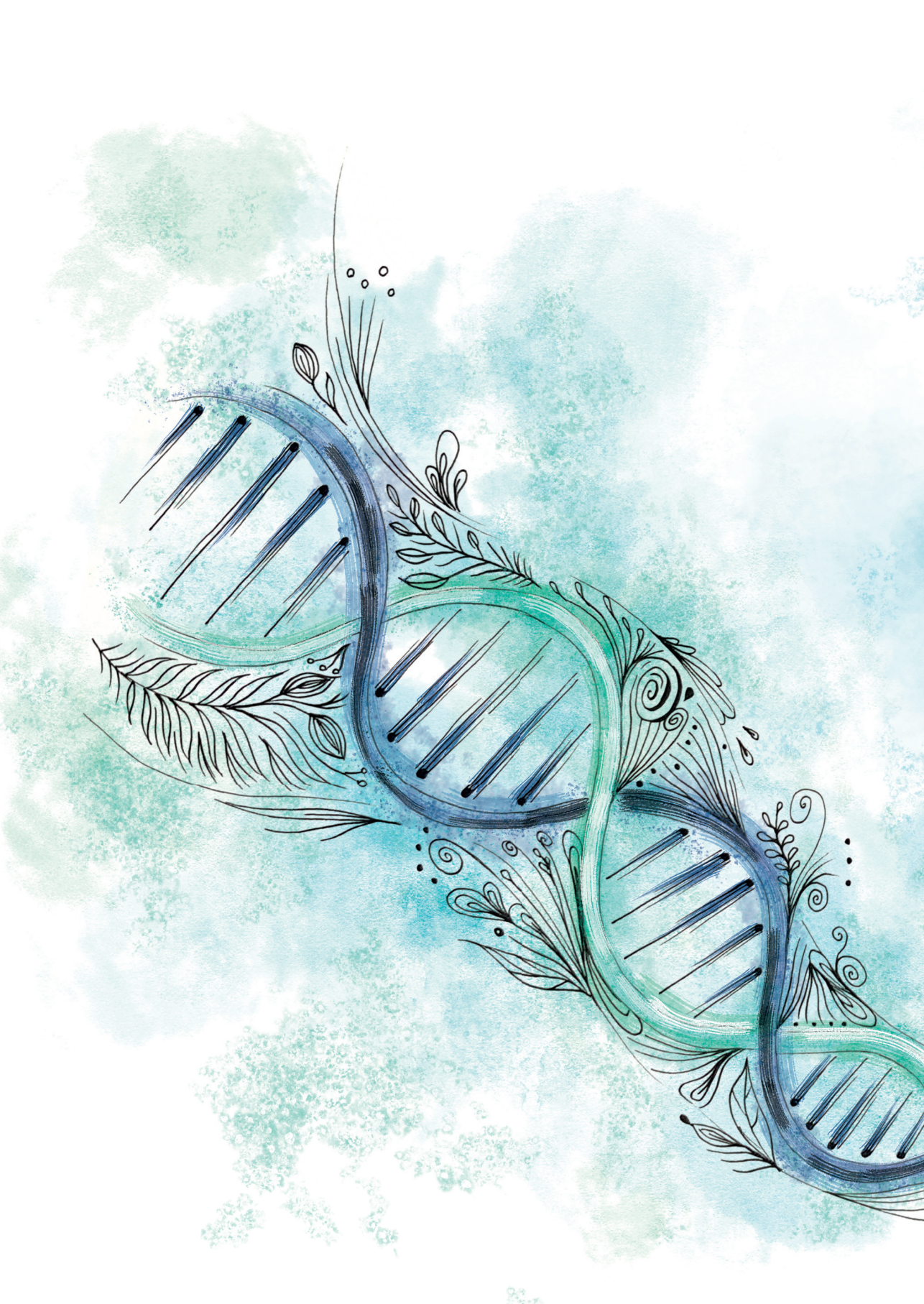

CHAPTER 2

NeuroD factors discriminate mineralocorticoid from glucocorticoid receptor DNA binding in the male rat brain

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Abstract

In the limbic brain, mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) both function as receptors for the naturally occurring glucocorticoids (corticosterone/cortisol), but mediate distinct effects on cellular physiology via transcriptional mechanisms. The transcriptional basis for specificity of these MR- versus GR-mediated effects is unknown. To address this conundrum we have identified the extent of MR/GR DNA binding selectivity in the rat hippocampus using chromatin immunoprecipitation followed by sequencing (ChIP-seq). We found 918 and 1450 non-overlapping bindings sites for MR and GR, respectively. Furthermore, 475 loci were co-occupied by MR and GR. *De novo* motif analysis resulted in a similar binding motif for both receptors at 100% of the target loci, which matched the known glucocorticoid response element (GRE). In addition, the Atoh/NeuroD consensus sequence was found in co-occurrence with all MR-specific binding sites, but was absent for GR-specific or MR-GR overlapping sites. bHLH family members Neurod1, Neurod2 and Neurod6 showed hippocampal expression and were hypothesized to bind the Atoh motif. Neurod2 was detected at rat hippocampal MR binding sites, but not at GR-exclusive sites. All three NeuroD transcription factors acted as DNA-binding dependent coactivators for both MR and GR in reporter assays in heterologous HEK293 cells, likely via indirect interactions with the receptors. In conclusion, a NeuroD family member binding to an additional motif near the GRE seems to drive specificity for MR over GR binding at hippocampal binding sites.

Introduction

The endogenous glucocorticoid hormone of the rat, corticosterone, has a profound action on the brain. This action is mediated in a complementary manner by mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), which are unevenly distributed over the brain, but co-expressed in abundance in the hippocampus (1). The high affinity MRs are already substantially occupied with low corticosterone levels (2). In the initial response to stress, these MRs play a crucial role in retrieval of stressful information and the selection of an appropriate coping response (3-5). In contrast, the lower affinity GRs become activated only at higher corticosterone levels, around the peak of the circadian rhythm and during a stress response. GR activation promotes memory storage of the stressful experience (6, 7) and behavioral adaptation and recovery (1, 8).

Much progress has been made in understanding the cellular mechanism of these coordinated MR-GR mediated actions of corticosterone (9). Many of the effects depend on the transcriptional activity of the receptors. MR-mediated actions generally raise excitability in the hippocampus. In the most ventral part of the hippocampus corticosterone prolongs excitability via GR, providing an extended period for encoding of new information. In the dorsal pyramidal cells GR-mediated actions oppose those mediated by MR (10). That these MR- and GR-mediated effects of corticosterone are sometimes overlapping and in other processes are distinct is remarkable, given the large structural similarity between the two receptor types.

MR and GR are members of the nuclear receptor family, with a modular structure of an N-terminal domain (NTD), a DNA binding domain (DBD) and C-terminal ligand binding domain (LBD). Upon ligand binding, the receptors can dimerize and translocate to the nucleus, where they alter the transcription of their target genes. MR and GR can affect gene expression via tethering to other proteins such as AP-1 and NFκB (11), but in the hippocampus, at least under basal conditions, the main mechanism seems to be via direct DNA binding to the glucocorticoid response element (GRE) – palindromic sequences that are variations of AGAACANNNTGTTCT (12). Homo- as well as heterodimers of the receptors may occur (13, 14). The intrinsically unstructured NTD contains an Activator Function (AF)-1, and the LBD contains a ligand-dependent AF-2. Through these AF domains the receptors can interact with coregulators, which can modulate the transcriptional effects by histone modifying activity and recruitment/stabilization of the transcription factor complex (15). The fact that the two receptors are 94% identical in their DBD (16), suggests

that other mechanisms must exist that confer transcriptional specificity underlying the differential effects of MR/GR.

It has remained elusive to what extent genomic targets of MR and GR overlap and what determines the specificity of MR and GR DNA binding. We previously identified genomic loci for GR, using chromatin immunoprecipitation-sequencing (ChIP-seq) after a single injection of corticosterone (12). In the current study we aimed to characterize mechanisms that confer MR/GR specificity by directly comparing their genomic binding sites in the same tissue. Our findings suggest that interactions between MR/GR and DNA-binding transcription factors from the NeuroD family are responsible for MR-selective signaling in the limbic brain, and that NeuroD factors are able to potentiate transcriptional activity of both receptor types *in vitro*.

Material and methods

In vivo experiment

For the ChIP-seq experiment, adult male Sprague Dawley rats (Harlan, The Netherlands) were housed on a 12:12-hour light/dark cycle (lights on 7:30 AM) with food and water *ad libitum*. ChIP-seq with MR, GR or control immunoglobulin G (IgG) antibody was performed on hippocampal tissue of 3 day adrenalectomized animals 60 min after a single intraperitoneal injection of 300 or 3000 μg/kg corticosterone as a 2-hydroxypropyl-β-cyclodextrin complex (CORT-HBC), as described (12). ChIP-seq was done on pooled tissue from 6 animals per treatment, which was redivided leading to 4 technical ChIP replicates for both MR and GR. All experiments were performed according to the European Commission Council Directive 2010/63/EU and the Dutch law on animal experiments and approved by the animal ethical committee from Leiden University.

ChIP-sequencing analysis and motif search

The MR binding data were generated and analyzed in parallel with the previously published data for GR (12). Illumina Genome Analyzer 35 bp single end reads were uniquely mapped to the rattus norvegicus genome version 4 (rn4). Peaks were called using Model-based Analysis of ChIP-Seq (MACS) (17) with the IgG antibody binding dataset as the background. Binding sites were considered overlapping if more than 4 bp were shared. Data were visualized by uploading wiggle files to Integrative Genomics Viewer (IGV) (18). Using the annotate peak function of HOMER, binding sites were associated

to their nearest gene (19). The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for gene ontology analysis (20). Binding sequences were analyzed for the presence of *de novo* motifs using Multiple Expectation maximization for Motif Elicitation (MEME) (21). The motif size was set from 6 bp min to 20 bp max, searching also the reverse complement, with a maximum of 10 output motifs, using random shuffled input sequences as background model. Enriched motifs were compared against the JASPAR vertebrate database of known motifs using TOMTOM motif comparison tool. Analysis of Motif Enrichment (AME) was used for enrichment analysis of known motifs in MR-exclusive relative to GR-exclusive binding sequences, and Motif Alignment & Search Tool (MAST) for directed search of motifs of interest, under default settings (21).

ChIP-qPCR validation

For binding site validation we performed ChIP-qPCR on hippocampal tissue of adrenally intact rats sacrificed at the time of their endogenous corticosterone peak. Antibodies used are listed in **Supplemental Table 1**. Protease inhibitors (Roche) were added to all buffers during tissue processing and the ChIP procedure. Hippocampal hemispheres were fixated with 1% formaldehyde for 12-14 min and were homogenized in Jiang buffer (0.32 M sucrose, 5 mM CaCl2, 3 mM Mg(Ac)2, 0.1 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.1% Nonidet P (NP)-40) using a glass douncer (Kimble-Chase). Following steps were performed in NP buffer (150 mM NaCl, 50mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100). Chromatin was fragmented by sonication for 32 min, 30 sec ON/30 sec OFF cycles, using a Bioruptor Pico (Diagenode). Three processed hippocampal hemispheres were pooled and redivided to perform a ChIP for both MR and Neurod2. From each chromatin sample an aliquot was taken as input material, to be able to calculate the percentage of immunoprecipitated DNA. Chromatin (500 µL) was incubated overnight with 6 µg antibody, after which 20 µL protein A Sepharose beads (GE healthcare) were added for 2.5 hours. After several washing steps (**Supplemental Methods**), antibody-bound DNA was eluted from the beads using 10% Chelex 100 (Bio-Rad), further purified by phenolization and dissolved in 50 µL H_2O. qPCR was performed on 4x diluted ChIP samples according to the protocol described below. Primers were designed to span the GRE of the discovered binding sites, and are listed in **Supplemental Table 2**.

Reporter assays

For mechanistic insights into the effect of NeuroD factors on MR/GR promoter activity, we performed luciferase reporter assays. HEK293 cells (human embryonic kidney, female)

were cultured in Dulbecco's Modified Eagle Medium with GlutaMax (Gibco) containing 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) and 10% fetal bovine serum (FBS, PAN-Biotech), at 37°C under 5% CO_2. For the reporter assays, cells were seeded in a 24-wells plate at a density of 80,000 cells/well and grown in medium supplemented with charcoal stripped FBS (Sigma) to exclude cortisol action from the serum. Cells were transfected on day 2 with luciferase construct (TAT1-Luc or TAT3-Luc: 25 ng/well; GRE-At, MRE-At or GRE-MutAt: 30 ng/well), expression vector for one of the receptors (MMM, ∆MM, MM∆, GGG, ∆GG, GG∆: 10 ng/well), pCMV-Myc-Neurod1/2/6 (0-1-3-10-50-100 ng/well), completed with pcDNA3.1 to a total of 300 ng/well and 1.25 µL/well FuGENE (Promega) in unsupplemented DMEM. Renilla luciferase was used to correct for transfection efficiency (1 ng/well, pRL-CMV, Promega). On day 3, cells were stimulated with corticosterone (10^{-7} M or at indicated concentrations, Sigma) dissolved in ethanol, diluted in medium with a final concentration of 0.1 % ethanol. After 24 hours the cells were washed with phosphate buffered saline and reporter protein was measured using the Dual-luciferase Reporter Assay System according to the manufacturer's instruction (Promega). Briefly, 100 µL lysis buffer was added and after 10 min 10 µL lysate was transferred into a half area 96-wells plate. Luciferase levels were quantified with 25 µL luciferase assay substrate at 570 nm; subsequently Renilla signal was measured at 470 nm after the addition of 25 µL Stop & Glo at a SpectraMax L microplate reader (Molecular Devices). All data are presented as mean ± SEM. Reporter assays were done in triplicates, and repeated at least once.

Plasmids

The GRE-At and GRE-MutAt luciferase constructs were created by inserting a 36-bp fragment containing a perfect palindromic GRE plus the Atoh1 motif or the GRE with a scrambled motif in the XhoI site of a pGL4.10[luc2] vector (Promega). Inserts were GRE-At: ctcgagGATGGCAGATGGAGCTAAGAACAGAATGTTCTATAActcgag and GRE-MutAt: ctcgagGATGGAGCGGATAGCTAAGAACAGAATGTTCTATAActcgag. The MRE-At luciferase construct was created by inserting a 35-bp endogenously found MR binding site containing a more degenerate GRE plus the Atoh1 motif in the NheI/BglII site of the same pGL4 vector. MRE-At insert was: gctagcGCACACAGATGAGTGGGGATCTGAATGTACTGTGGagatct. The pCMV-Myc-Neurod6 expression vector was kindly provided by Dr. Mitsuhiko Yamada (22). *Neurod1* and *Neurod2* were amplified from Sprague Dawley rat hippocampal cDNA using the primers forward 5'-CAGTAGTCGACCATGACCAAATCATACAGCGAG-3', reverse 5'-GTACTCTCGAGTGCCTCTAATCGTGAAAGATGG-3' and forward 5'-CAGTAGTCGACCATGCTGACCCGCCTGTT-3', reverse

5'-GTACTCTCGAGAGGTCTCAGTTATGGAAAAACGC-3' respectively and cloned in frame into the SalI/XhoI site of the same pCMV-Myc vector to gain pCMV-Myc-Neurod1 and pCMV-Myc-Neurod2. Expression vectors for rat receptors 6RMR (MMM), 6RGR (GGG) and their corresponding truncated receptors 6RMR/596C (ΔMM), 6RMR/N689 (MMΔ), 6RGR/407C (ΔGG), 6RGR/N525 (GGΔ), and TAT1/3-Luc reporters were kindly provided by Dr. David Pearce (23).

Real-time quantitative PCR

To validate the NeuroD factor expression in the rat brain, we performed RT-qPCR measurements on Sprague Dawley tissue. Hippocampal hemispheres were homogenized in TriPure (Roche) by shaking the tissue with 1.0 mm diameter glass beads for 20 s at 6.5 m/s in a FastPrep-24 5G instrument (MP Biomedicals). Total RNA was isolated with chloroform, precipitated with isopropanol, washed with 75% ethanol and resuspended in nuclease-free H_2O. The purity and concentration of the RNA samples were measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA was reverse transcribed from 1 µg RNA using random hexamers and M-MLV reverse transcriptase (Promega), incubated for 10 min at 25°C, 50 min at 45°C and 10 min at 70°C. RT-qPCR was performed in duplo on 10x diluted cDNA (5 ng/µL) with final primer concentrations of 0.5 µM using GoTaq qPCR master mix (Promega) in a CFX96 real-time PCR machine (Bio-Rad). The program consisted of 40 cycles of 10 s at 95°C and 30 s at 60°C, followed by a melting curve generation from 65°C to 95°C in steps of 0.5°C. Primer sequences are listed in **Supplemental Table 2**.

Allen Brain Atlas correlations

Lists of MR-exclusive, MR-GR overlapping and GR-exclusive genes corresponding to the intragenic and distal promoter (up to -5000 bp) ChIP-seq binding sites were evaluated for their co-expression with each studied NeuroD factor, using the mouse brain gene expression data from the Allen Brain Atlas (24). Pearson's correlation coefficient was used as a measure of similarity between the expression profile of the seed genes (*Neurod1*, *Neurod2* and *Neurod6*) and every gene in the three aforementioned lists within an anatomical region of interest (25). Correlations were calculated in the hippocampus, and its subregions cornu ammonis (CA)1 to CA3 and the dentate gyrus (DG) as well as the striatum. In order to assess the strength of the association between each gene list and a seed gene, we used a one-sided Wilcoxon rank-sum test.

Data deposition

ChIP-seq data have been submitted to the European Nucleotide Archive (ENA) and are publicly available under accession number PRJEB18916.

Results

MR-GR binding site overlap

ChIP-seq on hippocampus chromatin with MR and GR antibodies resulted in the generation of 1.3-1.9x10^7 reads per sample. After uniquely mapping 66.6-83.5% of these reads to the rat genome (rn4), MACS peak calling with a false discovery rate (FDR) cut-off at 13.5% (conform Polman et al., 2013; **Supplemental Figure 1A**) resulted in 768 MR sites in the animals injected with 300 µg/kg (MR300), and 1465 MR sites and 2460 GR sites in the animals injected with 3000 µg/kg (MR3000 and GR3000).

We computed the overlap in binding site genomic coordinates for MR and GR (**Figure 1A**). Additional filtering of MR- and GR-exclusive sites demanded total absence of any peak (the MACS lists including those peaks with an FDR above 13.5%) at the same locus in the GR and MR data, respectively. This resulted in 918 MR-exclusive sites (combined from the MR300 and MR3000 dataset), 475 MR-GR overlapping sites and 1450 GR-exclusive sites (**Supplemental Table 3**). These correspond to 45.9% of the total MR sites and 58.9% of the total GR sites being non-overlapping. ChIP-seq traces of an MR-exclusive, MR-GR overlapping and GR-exclusive peak are shown in **Supplemental Figure 2**. The distribution of sites relative to nearest genes is similar for these subsets, with approximately 40-45% of the binding sites located within promoters and genes - mainly in introns (**Supplemental Figure 1B**). Limited overlap was found between the MR binding sites for the two different dosages, as only 30.6% of the MR300 sites were also found in the MR3000 dataset.

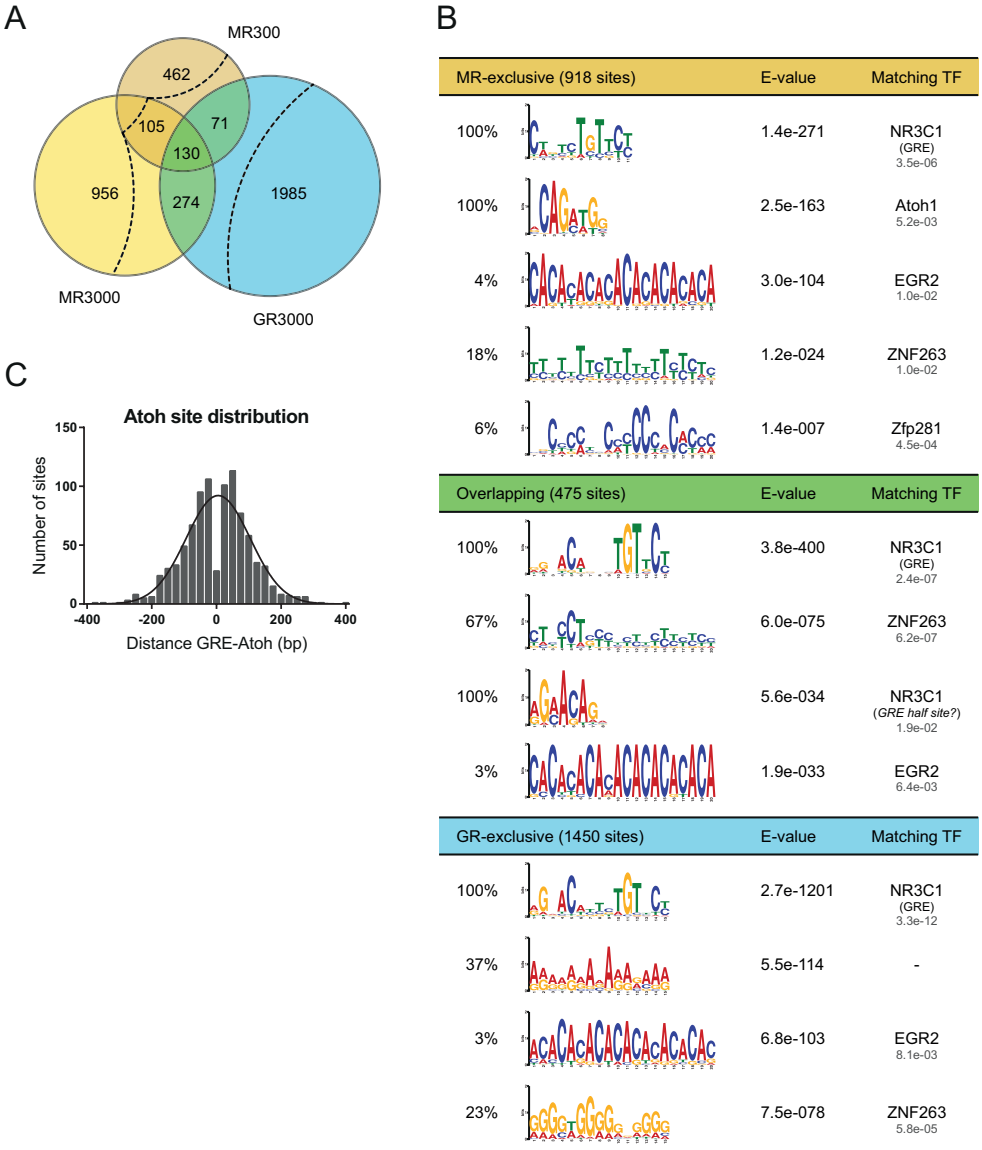

Figure 1. ChIP-seq binding site analysis. **A)** Overlap of MR and GR binding sites in the rat hippocampus, from animals injected with 300 µg/kg (MR300) or 3000 µg/kg (MR3000 and GR3000) corticosterone. Dashed lines represent the additional filtering of non-overlapping sites demanding total absence of any peaks in the other receptor dataset, leading to 918 MR-exclusive (combined from MR300 & MR3000), 475 overlapping and 1450 GR-exclusive sites. **B)** *De novo* motif analysis of MR-exclusive, overlapping and GR-exclusive binding sites. Discovered motifs are depicted with their E-value (MEME) and the highest ranked matching transcription factor (TF). Listed TFs are followed by the E-value (TOMTOM) for the motif comparison. **C)** Distribution of distance between GRE and Atoh motifs over 25 bp bins, including a normal curve. Depletion of the histogram bin around zero is due to the minimum distance of 8bp as calculated from the center of the GRE to the center of the Atoh motif.

Validation of MR binding sites

The GR binding sites were thoroughly validated before (12). We performed ChIP-qPCR measurements for MR in the hippocampus of adrenally intact animals sacrificed at the time of their endogenous corticosterone peak. MR binding was detected at all tested MR-exclusive sites, whereas no MR signal was found at any of the GR-exclusive sites (**Figure 2A**). This demonstrates that the selectivity found in the pharmacological ChIP-seq experiment, also occurs in a physiological context.

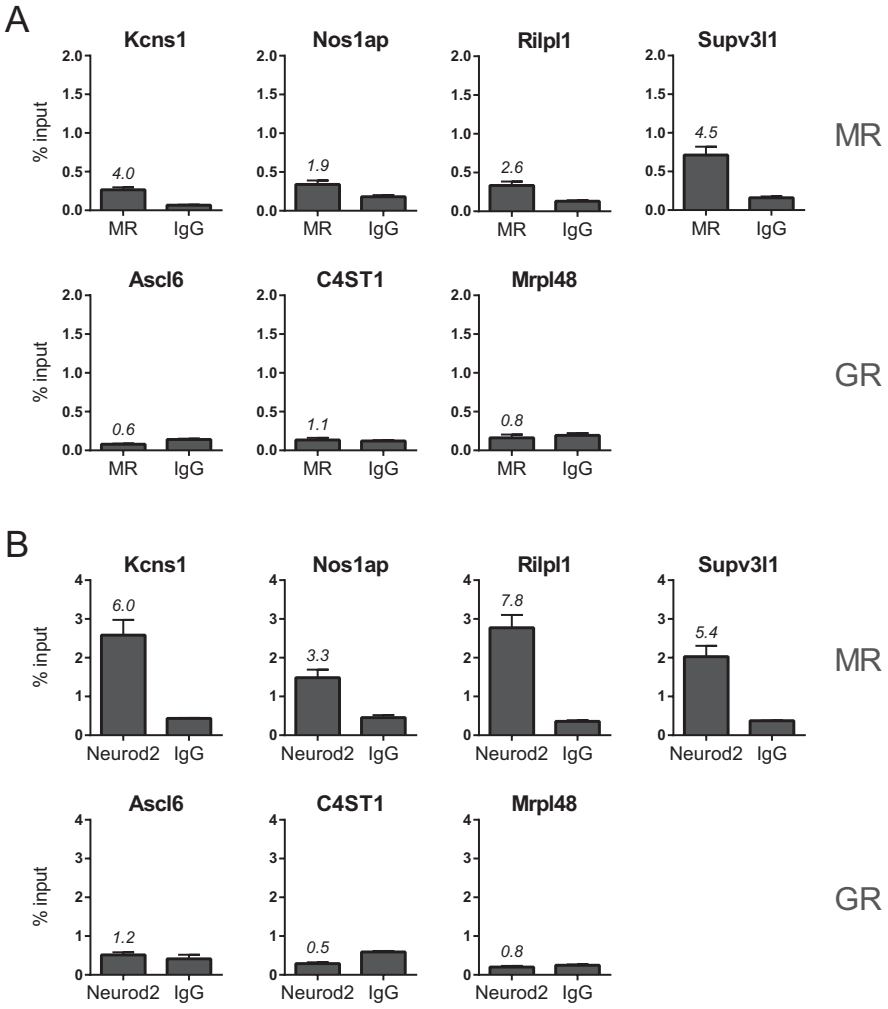

Figure 2. ChIP-qPCR validation of **A)** MR (n=5) and **B)** Neurod2 (n=6) binding to a subset of MR-exclusive and GR-exclusive binding sites. Numbers indicate the fold induction over IgG background.

Processes associated with MR and GR target genes

The biological relevance of the hippocampal binding sites was examined by gene ontology enrichment analysis of target genes, under the assumption that expression of MR/GR bound genes will be regulated by the receptor. Intragenic and upstream (up to -5kb) binding sites were annotated to generate lists of MR-exclusive, overlapping and GR-exclusive target genes (**Supplemental Table 4**). Functional annotation clustering using DAVID showed enrichment of brain-related terms, such as *Regulation of cell projection assembly* (MR), *Synapse, Regulation of synaptic plasticity* (overlapping) and *Cell/neuron projection, Synaptic vesicle* (GR) (**Supplemental Table 5**). Interestingly, for those genes linked to specific MR binding there was enrichment for *Sodium channel activity*, *Calcium ion transport* and *Ion transport, voltage-gated channel activity*. Another term specific for MR-exclusive target genes was *Cell adhesion*. Furthermore the annotated GR-exclusive target genes were associated with *Apoptosis* and *Response to oxidative stress*.

An additional motif was found near MR-exclusive sites

To explore the biological mechanism underlying MR/GR-selective binding, we performed *de novo* motif analysis on the binding site sequences. For the MR, as well as the overlapping and GR datasets, all sites contained a glucocorticoid response element (GRE) (**Figure 1B**). This is in contrast to the aldosterone-induced MR cistrome in a human renal cell line, where the majority of binding sites lack a GRE (26). The MR-exclusive sites had a more degenerate GRE (lower probability of bases) than the GR-exclusive sites. All subsets also contained a motif that matched the ZNF263 binding site, which was present in 18-67% of the sequences. The MR-GR overlapping sites all contained a motif that resembles a GRE half site, suggestive of concomitant dimeric and monomeric (or multimeric) binding of the receptors.

Interestingly, we found a distinct motif near the MR-exclusive sites, that was not enriched near the GR-exclusive or overlapping sites. This additional motif was present in 100% of the MR sites and matched to the Atoh1 binding sequence in the motif database. In a directed search, the Atoh1 motif was also enriched in MR over GR binding (AME, $p = 1.11 \times 10^{-24}$), although in individual cases we observed this site near GR-bound GREs (MAST, 1% of the GR-exclusive sites). The distance between the GRE and Atoh motif was normally distributed (**Figure 1C**) and independent of their respective orientation/strand (in or out of phase) or the binding site relative to genes (intergenic versus intragenic) (**Supplemental Figure 1C**). We supposed that another protein binding to this Atoh site can drive MR-specific binding.

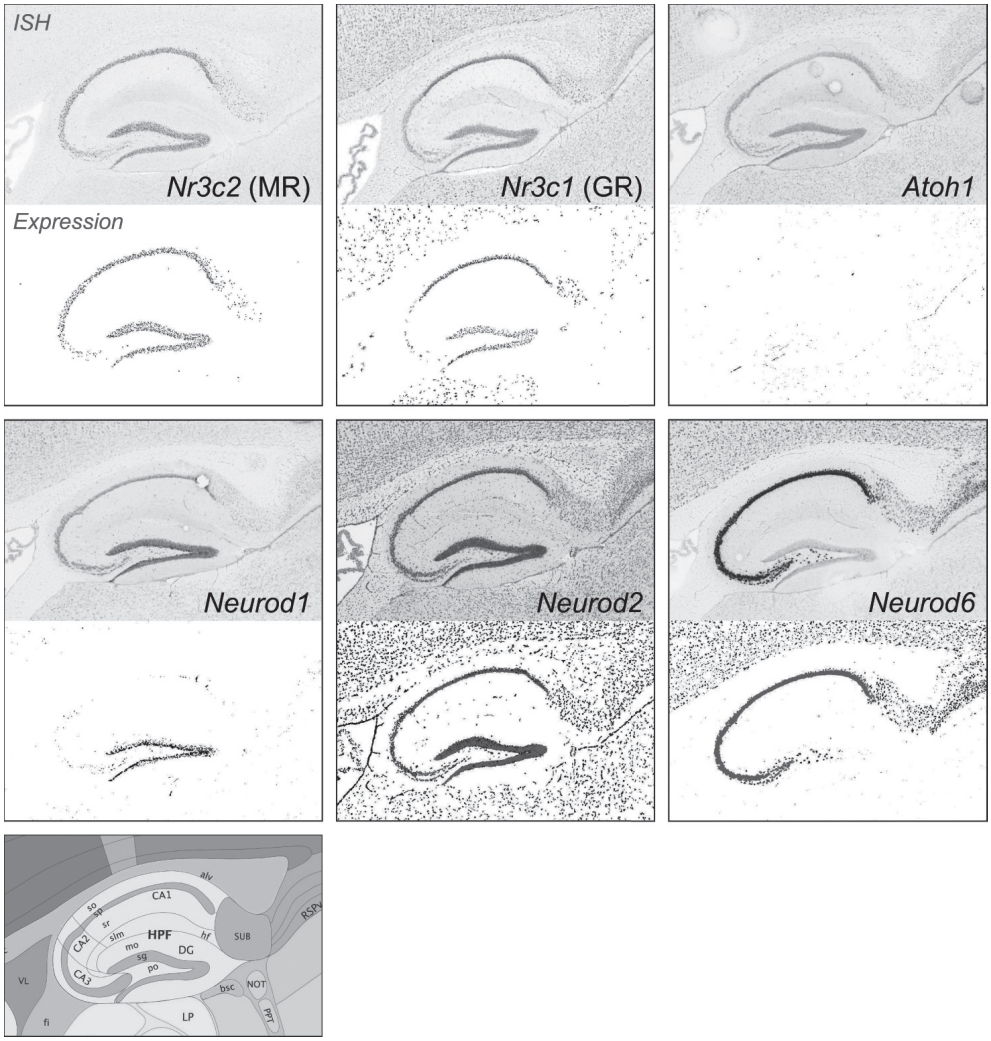

Figure 3. Expression of MR, GR, Atoh1 and NeuroD family members in the adult mouse hippocampus, with the corresponding reference atlas. Visualizations of the sagittal *in situ* hybridization (ISH) experiments and corresponding background subtracted signals (Expression) from the Allen Brain Atlas (24). Experiment_position numbers of depicted images are listed in the Supplemental Methods.

NeuroD family members as candidate binders

According to the Allen Brain Atlas (24), *Atoh1* is not expressed in the mouse hippocampus (**Figure 3** and **Table 1**) and is therefore not considered a candidate to bind the MR-specific motif found in the hippocampal ChIP-seq dataset. Atoh1 belongs to the basic helix-loop-

helix (bHLH) family of transcription factors (27). Brain-specific family members *Neurod1*, *Neurod2* and *Neurod6* do show evident hippocampal expression (**Figure 3**) and have been shown to bind the identified CAGATGG motif (28-30). We validated the very low expression levels (or absence) of *Atoh1* and expression of the three NeuroD genes in the rat hippocampus by RT-qPCR (**Table 1**) and hypothesized (one of) these corresponding proteins could be responsible for the binding site selectivity for MR.

Table 1. Overview of Atoh1 and NeuroD family members and validation of mRNA expression levels in rat hippocampus.

Protein	Synonyms	Expression peak	Adult hippocampal expression		
			Subregion	ABA	Ct
Atoh1	Hath1, Math1, bHLHa14	Early embryonic	-	0.24	>33.0
Neurod1	BETA2, BHF-1, Neurod, bHLHa3	E16-P0 *	Both CA & DG (higher in DG)	1.41	23.2
Neurod2	Ndrf, bHLHa1	Stable throughout development *	Both CA & DG	10.41	22.0
Neurod3	Neurog1, AKA, Math4C, bHLHa6, Ngn1	Early embryonic	-	0.29	-
Neurod4	AI846749, ATH-3, Atoh3, Math3, bHLHa4	Early embryonic	-	0.12	-
Neurod5	Atoh6	-	-	-	-
Neurod6	Atoh2, Math2, Nex, Nex1m, bHLHa2	P5 *	CA1-CA3	11.73	21.0

The effect of Neurod1, Neurod2 and Neurod6 (grey rows) on glucocorticoid signaling was studied *in vitro*. ABA = Allen Brain Atlas, raw expression value in adult mouse hippocampal formation, β-actin = 21.17; as a reference MR = 0.68, GR = 2.18. The threshold cycle (Ct) values represent RT-qPCR measurements on 5 ng/μL cDNA, Sprague Dawley rat whole hippocampus, β-actin = 17.8. *(50)

By ChIP-qPCR we demonstrated Neurod2 binding at the same sites at which we validated MR binding (**Figure 2B**). It was however absent from GR-exclusive loci. This gives a proof of concept that Neurod2 might be binding to the Atoh site *in vivo*. While Neurod2 was selected based on the availability of ChIP-grade antibodies, this result does not exclude involvement of Neurod1 or Neurod6 in MR-selective signaling.

In vivo co-expression of NeuroD factors with putative MR/GR target genes

To get an indication if the other two NeuroD factors could be (co-)responsible for the MR-selective binding *in vivo*, we examined to what extent they are co-expressed with putative MR/GR target genes (as defined by intragenic or up to -5kb binding of MR or GR). We assessed the spatial co-expression of the MR, overlapping and GR target gene lists with each of the NeuroD family members based on their expression patterns across the brain using data from the Allen Brain Atlas (24). The MR targets had a stronger co-expression with *Neurod6* than the overlapping or GR targets, while for *Neurod2* there was no difference between the three lists and the *Neurod1* spatial correlation was highest for the GR targets (**Supplemental Figure 3**). This could argue for Neurod6 as an *in vivo* determinant of MR-selective signaling. Nevertheless, all three NeuroD factors correlated strongly with the expression of MR-exclusive targets and were subsequently studied *in vitro*.

NeuroD family members potentiate MR/GR transactivation

The putative role of Neurod1, Neurod2 and Neurod6 in MR-specific signaling was further studied in reporter assays in HEK293 cells. All three proteins potentiated MR, but unexpectedly also GR transactivation upon corticosterone treatment on a luciferase construct containing a GRE plus the additional Atoh motif in its promoter (GRE-At), by approximately 4-fold and 7- to 9-fold, respectively (**Figure 4**). This effect was not observed at a control construct lacking the Atoh binding site (GRE-MutAt), and the NeuroDs could not enhance reporter expression without hormone stimulation. The NeuroD factors thus acted as MR/GR transcriptional coactivators via the identified Atoh motif. For Neurod6, a clear dose-response curve was observed for transfection with increasing doses of expression vector (**Supplemental Figure 4A**). We further tested a reporter driven by a more degenerate GRE, as found for the MR-exclusive sites (**Figure 1B**), combined with the additional Atoh site (MRE-At). The receptors were less efficient in stimulating this luciferase promoter and the NeuroD effect also did not differ for MR and GR on this reporter (**Supplemental Figure 4B**).

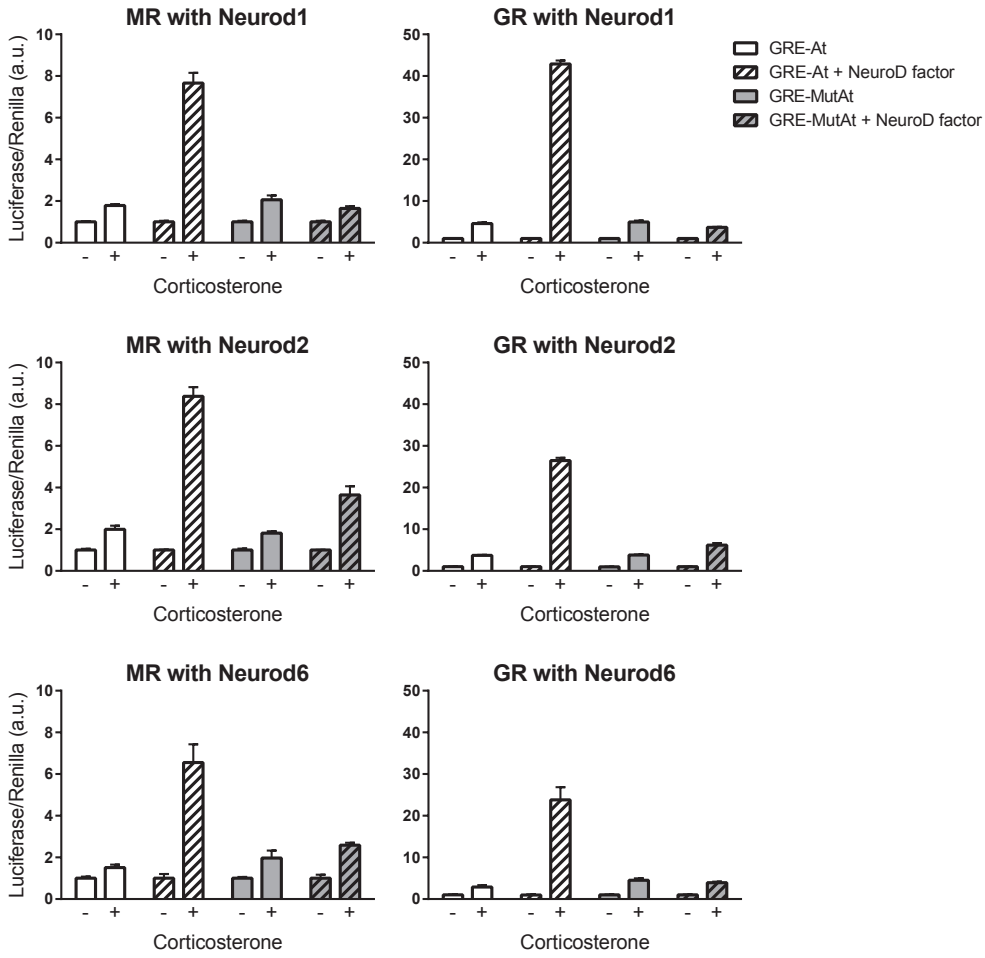

Figure 4. Potentiation of MR and GR transactivation by NeuroD family members on a luciferase construct containing a perfect GRE plus the additional MR-exclusive motif. HEK293 cells were transfected with MR or GR; the GRE-At or GRE-MutAt luciferase constructs; Neurod1, Neurod2 or Neurod6 (10 ng/well) and stimulated with corticosterone (10^{-7} M). Non-stimulated cells were normalized to 1. a.u. = arbitrary unit

NeuroD family members increase mainly the maximum transcriptional effect

As the mechanism of action of a receptor modulator can be deduced from both the change in maximum effect, as well as the ligand concentration needed for 50% of this effect (EC50) (31), we generated corticosterone dose-response curves with and without co-transfecting

Neurod6. The maximum MR/GR effect was increased by Neurod6 presence over the whole concentration range that activates the receptor (data not shown), as was seen before by increased luciferase expression at saturating corticosterone concentrations of 10^{-7} M (**Figure 4**). Besides, the EC50 was not changed for MR ($2.24\pm0.06 \times 10^{-10}$ M versus $1.89\pm0.05 \times 10^{-10}$ M), while the GR showed a slightly decreased EC50 upon Neurod6 addition ($1.03\pm0.05 \times 10^{-8}$ M versus $5.29\pm0.02 \times 10^{-9}$ M) (**Figure 5A**).

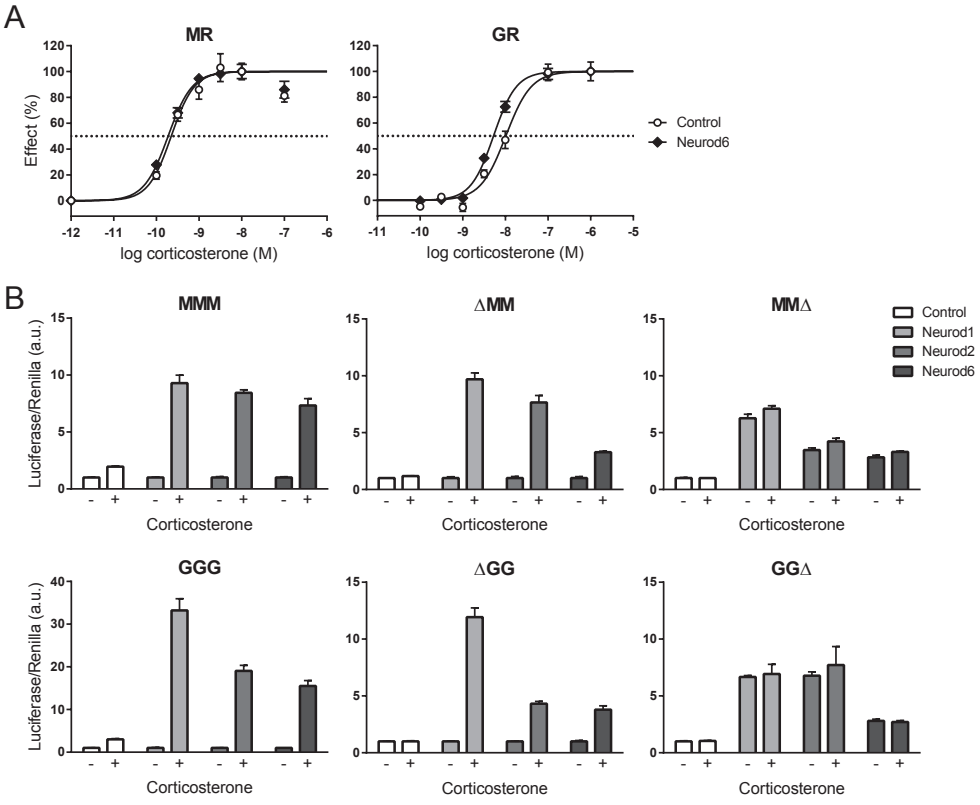

Figure 5. NeuroD increases the maximum MR/GR effect via an indirect mechanism of action. **A)** Dose-response curves for corticosterone stimulation of MR and GR in absence and presence of Neurod6, to determine the effect on EC50. The luciferase activity is presented as percentage of the maximum effect. Sigmoidal curves were fit by non-linear regression using a variable slope model. **B)** Effect of NeuroD factors on truncated receptors. HEK293 cells were transfected with full MR or GR (MMM, GGG) or variants lacking the N-terminus (ΔMM, ΔGG) or C-terminus (MMΔ, GGΔ); the GRE-At construct; Neurod1, Neurod2 or Neurod6 (10 ng/well) and stimulated with corticosterone (10^{-7} M). All non-stimulated cells were normalized to 1; for the constitutively active MMΔ and GGΔ luciferase levels were normalized to non-stimulated control cells. a.u. = arbitrary unit

NeuroD family members interact with both N-terminal and C-terminal domain lacking receptors

To further investigate the mechanism of interaction between the MR/GR and NeuroD factors, reporter assays were performed using truncated receptors (**Figure 5B**). The transactivation by receptors lacking the LBD (MM∆ and GG∆) could be potentiated by the different NeuroDs, although to a lesser extent than for the full-length receptors (MMM and GGG). The potentiation by NeuroDs was also seen without hormone treatment for these constitutively active receptors lacking the LBD. Besides, the NeuroDs could also increase transcriptional activity of the receptors that did not have an N-terminal domain (∆MM and ∆GG). For MR the NeuroD potentiation of the truncate was comparable to that for the full length receptor, but for GR the enhancement relative to non-stimulated cells was less than half that of the full length receptor. Unexpectedly, the ∆MM and ∆GG were unresponsive to corticosterone treatment at this reporter, but we did confirm proper transactivation at TAT1-Luc and TAT3-Luc reporters (data not shown). This potentiation of both N- and C-terminal receptor truncations suggests that NeuroD factors have an indirect interaction with MR/GR.

Discussion

This study examined the overlap and specificity of MR versus GR regarding whole genome hippocampal binding sites. We found both MR-specific, GR-specific and joint sites, that all contained a GRE. Virtually all MR-specific sites had an Atoh consensus sequence within 400 bp of the GRE, whereas *de novo* motif analysis did not find this sequence near sites that showed GR occupancy (including overlapping sites). Neurod1, Neurod2 and Neurod6 are co-expressed with MR and/or GR in the principal hippocampal cell layers, and all could act as coactivators of both MR and GR in reporter assays.

The limited overlap found in MR and GR binding sites is in accordance with the distinct roles of the two receptors in the hippocampus (6, 10, 32). It should be noted however that the lower sequencing depth of our analysis might have precluded the detection of weaker binding sites. In addition, as we performed ChIP-seq on whole hippocampi, the small proportion of shared targets could also be a result of cell type specific MR/GR loci as a consequence of the differential MR and GR expression patterns throughout the hippocampal area. Co-expression of MR and GR is observed in the majority of CA pyramidal and dentate gyrus granular neurons, with the exception of CA3 pyramidal cells that have high MR but low GR levels (33). Besides GR is also expressed in glial cells (34, 35).

Limited overlap in the MR binding sites for the two different corticosterone doses (MR300 versus MR3000) could be explained partly by an insufficient depth of sequencing (limit of detection). In addition it might reflect different concentrations of activated MR in the nucleus, in combination with differential affinity of binding sequences for the receptor – even if the majority of MR likely was occupied by the lower dose. A recent study suggests that high receptor occupancy does not necessarily translate into high DNA binding, and MR can show circadian variation in target site occupancy (36). Differences in sensitivity between MR-expressing cell types might also be of relevance. A last possibility may be opening up of chromatin domains via GR, making GREs available for MR binding. In the same line, heterodimerization of MR and GR could play a role (36).

The additional, MR-selective motif could be bound by Neurod1, Neurod2 and Neurod6, as evidenced by response-element dependent transcriptional modulation. NeuroD proteins are members of the bHLH protein family and are known for their function in neuronal differentiation (28, 37). *Neurod1* knockout mice lack a dentate granule cell layer (38), and heterozygous *Neurod2* deficient mice show impaired contextual and cued freezing in a fear-conditioning task (29). Our binding sites were detected in adult rat hippocampal tissue, suggesting that the NeuroD factors not only regulate neuronal differentiation during development, but also can be crucial in later processes such as cell survival or retaining differentiation status. As the hippocampal dentate gyrus is the main site of adult neurogenesis (39), this might also provide a role for NeuroD factors in adulthood, although their expression is much wider than neurogenic zones. Furthermore, overexpression of Neurod2 in the ventral hippocampus has recently been shown to increase stress susceptibility in a chronic social defeat paradigm (40), posing a role for Neurod2 in depression.

Based on mouse brain expression data from the Allen Brain Atlas, we observed that *Neurod6* expression is restricted to the CA subregions of the hippocampus, while the lower *Neurod1* signal seems to be more pronounced in the DG (**Figure 3**). Furthermore, *Neurod2* expression is observed throughout the whole hippocampus and seems to be at levels similar to *Neurod6*, as we validated by RT-qPCR on rat hippocampal tissue. The three NeuroD proteins have a highly similar bHLH region (37), which makes it not surprising that all members can bind the additional Atoh motif derived from our ChIP-seq analysis and potentiate MR/GR transactivation in reporter assays. Based on our data we cannot pinpoint which of the family members is/are responsible for the MR specific binding, although Neurod2 was detected at rat hippocampal MR-exclusive sites (**Figure 2B**) and target gene correlations suggest that Neurod6 is also a likely candidate (**Supplemental**

Figure 3). We cannot exclude the possibility that another bHLH containing protein binds to the Atoh motif and drives the exclusive MR action. *Neurod1* or *Neurod2* deficient mice that also lack *Neurod6* have more severe brain abnormalities than the single mutants, indicating cooperation and/or partial redundancy (41, 42). A model in which Neurod1, Neurod2 and Neurod6 are each involved in MR-specific signaling within a certain subregion of the hippocampus might be considered.

The *in vivo* found MR-exclusive motif does not discriminate *in vitro* in reporter assays. This discrepancy could be explained by the possibility that in the luciferase assay the receptors use different intermediate transcriptional proteins than in the hippocampus. The observed coactivation of both N- and C-terminally truncated receptors implies that NeuroD family members interact via the transcriptional complex of MR/GR rather than directly with the receptors. A side note is that we cannot rule out interactions via the DBD or hinge region of the receptors. Nevertheless, the suggested indirect interaction is also supported by the fact that the Atoh motif was found at a variable distance up to 400 bp from the GRE. It is likely that the HEK293 cells lack or do contain other variants of the proteins that are crucial to mediate the NeuroD effect on selective MR transcriptional activity. For example, the pool of coregulators present in a cell is highly tissue-specific and can result in opposite effects on gene transcription (43). Also, bHLH protein heterodimerization partners might be responsible for an MR specific effect (27). Besides, as the chromatin landscape is a crucial determinant of a transcription factor cistrome (44), the lack of chromatin context in the luciferase assay might make it difficult to mimic the exact conditions of *in vivo* binding and transcription. Interestingly, Neurod1 itself can also induce chromatin remodeling and increase neuronal gene accessibility (45).

In lung fibroblasts, the Atoh1 motif was detected, although non-significantly, near GR-bound sequences (46). Directed motif search by MAST showed the presence of a Neurod2 binding site in 1% of our GR-exclusive sites, but the Atoh motif was clearly enriched in MR- over GR-exclusive sites using AME. It might be that a NeuroD factor through binding to the Atoh motif only excludes GR binding and subsequent transactivation when MR is present, which can be another reason that we do not find a difference in MR/GR potentiation *in vitro* when studying the receptors in isolation. In co-transfections of MR and GR combined with selective pharmacological activation, also both receptors were potentiated by Neurod6 (data not shown). Furthermore, the highly dynamic DNA binding kinetics of nuclear receptors are not supportive of a competition based mechanism (47, 48). A recent study also found motifs that were associated with absence of GR binding,

and proteins recognizing these sequences could indeed decrease GR occupancy and transactivation (49).

In conclusion, we identified a motif that is associated with MR-selective signaling in the rat hippocampus. NeuroD factors could bind this motif and via indirect interactions were found to potentiate the MR/GR transcriptional activity in HEK293 cells. The data support a model in which NeuroD factors stabilize MR binding *in vivo* by interacting with cell specific components of the MR-associated transcriptional complex. Further elucidation of distinct MR/GR downstream pathways will enable us to more specifically target aspects of glucocorticoid signaling for treatment of stress-related disorders.

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Supplemental data

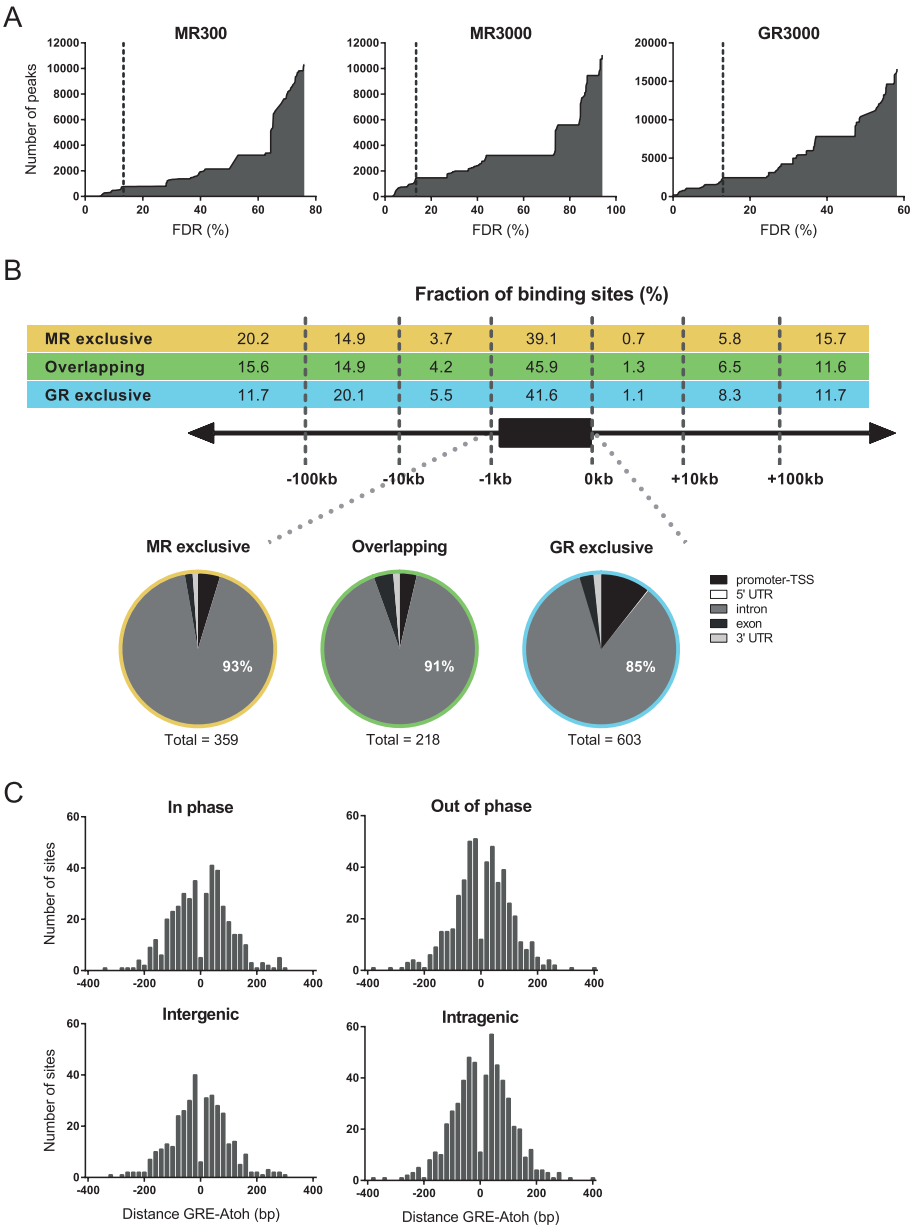
Supplemental Methods

ChIP washing buffers

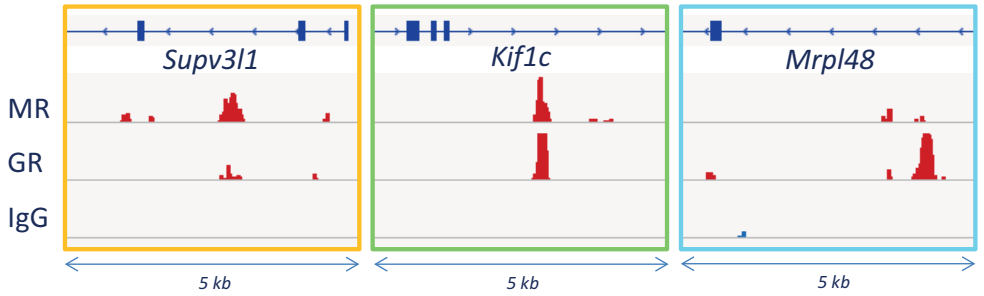
Low salt wash buffer (1x)
150 mM NaCl, 20 mM Tris-HCl pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100
High salt wash buffer (1x)
500 mM NaCl, 20 mM Tris-HCl pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100
LiCl wash buffer (1x)
10 mM Tris-HCl pH 8.1, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% deoxycholate
TE wash buffer (2x)
M Tris-HCl pH 8.0, 1 mM EDTA

Allen Brain Atlas experiment_position numbers used for Figure 2

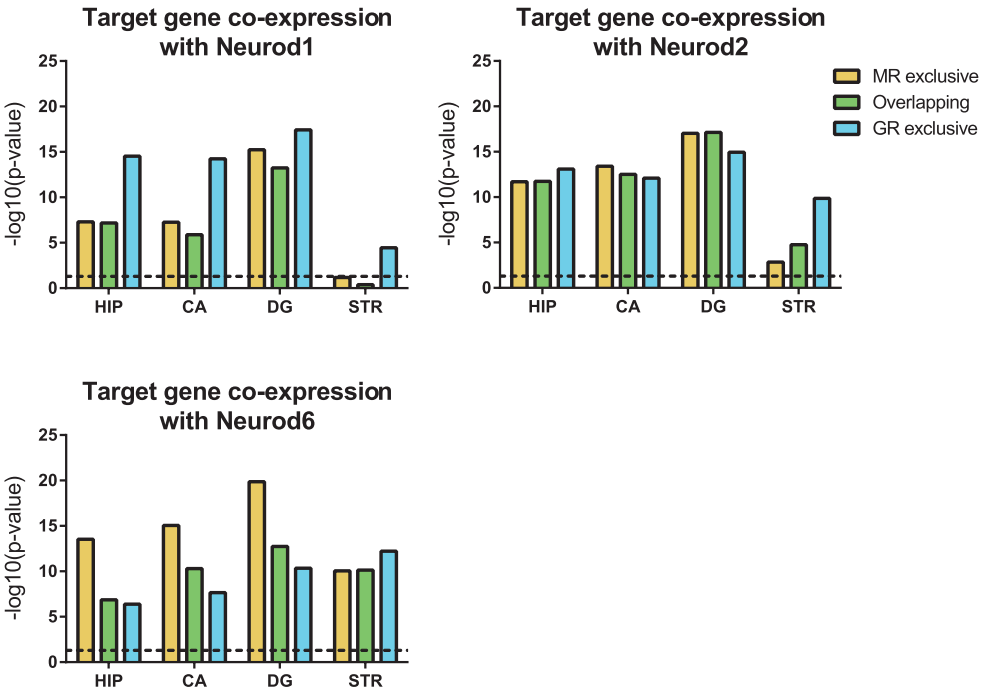
Nr3c2 (MR): 731_91
Nr3c1 (GR): 728_102
Atoh1: 75826683_96
Neurod1: 79632311_96
Neurod2: 70437810_98
Neurod6: 79544834_101



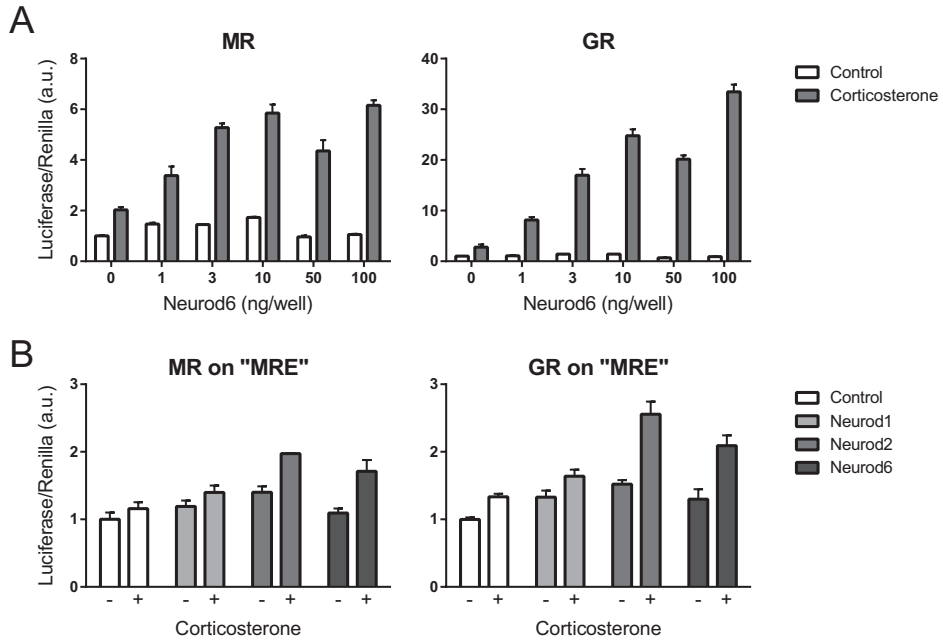
Supplemental Figure 1. ChIP-seq binding site analysis. **A)** FDR distribution across called peaks, with evident gaps from 13.33-28.12% for MR300, 13.49-26.88% for MR3000 and 12.98-24.08% for GR3000 datasets. Based on this 13.5% was set as FDR cutoff. **B)** Genomic distribution of MR, overlapping or GR binding sites relative to the nearest gene. Insets show detailed locations of intragenic (promoter-TSS, intron, exon, UTR) binding sites. Promoter-TSS is defined as -1kb to +100bp. **C)** Effect of relative motif orientation and location relative to genes on distribution of distance between GRE and Atoh motifs. Motifs were found both on the same strand (in phase) or on the opposite strands (out of phase). TSS = transcription start site, UTR = untranslated region



Supplemental Figure 2. IGV browser screenshots showing examples of an intragenic MR-exclusive (*Supv3l1*), overlapping (*Kif1c*) and GR-exclusive (*Mrpl48*) binding site.



Supplemental Figure 3. Target gene list co-expression. Correlation of target genes with NeuroD family member expression for the whole hippocampus (HIP), CA and DG subregions and striatum (STR) as control region. Logarithm of the Wilcoxon rank-sum test p-value. Dashed lines represent the significance level.



Supplemental Figure 4. **A)** Dose-response curve of Neurod6 transfection on GRE-At construct. **B)** Effect of NeuroD on a luciferase construct containing a more degenerate GRE plus the additional MR-exclusive motif. HEK293 cells were transfected with MR or GR; the MRE-At luciferase construct; Neurod1, Neurod2 or Neurod6 (10 ng/well) and stimulated with corticosterone (10^{-7} M). Luciferase levels were normalized to non-stimulated control cells.

Supplemental Table 1. Antibodies used for ChIP.

Target	Antigen sequence	Name	Manufacturer, catalog number	Species raised in, clonality	RRID
MR	Amino acids 1-300 of human MR	MR antibody (H-300) X	Santa Cruz Biotechnology, sc-11412X	Rabbit polyclonal IgG	AB_2155949
GR	Amino acids 121-420 of human GR	GR antibody (H-300) X	Santa Cruz Biotechnology, sc-8992X	Rabbit polyclonal IgG	AB_2155784
Neurod2	Synthetic peptide of human NeuroD2 residues	NeuroD2 antibody [EPR5135]	Abcam, ab109406	Rabbit monoclonal IgG	AB_10866309
IgG	No known specificity	Isotype control	Abcam, ab37415	Rabbit polyclonal IgG	AB_2631996

All antibodies were used in a dilution of 6 µg/500 µL.

Supplemental Table 2. Primer sequences used for ChIP-qPCR validation (upper list) and RT-qPCR on rat hippocampal cDNA (lower list). Mouse intestinal cDNA was used as a positive control to test the efficiency of Atoh1 primers.

Binding site	Nearest gene	Forward & reverse (5'>3')	Product length (bp)
MR300_116	*Kcns1*	GGCCTTAGTGAAGGAACCAGG ACTCACCATCTGCTCCTTGG	153
MR300_196	*Nos1ap*	GGTGTCTTTTTCTCTTCCCACAC AAAGATAAGCAGACCAACCCA	183
MR300_503	*Rilpl1*	CAGGCAGATGCCAGGCT CCCATGCCTGTTCCTCTAGT	106
MR3000_359	*Supv3l1*	TCTGTGTGTGACTGCCTGAC CTCTCAGGGCTTCCCTGTTT	111
GR3000_1726	*Ascl6*	CCTGCCAGGAGAGCAGATG TGTGCAGGAAGGCAAGTTCT	178
GR3000_193	*C4ST1*	ACCCTCTCTGAATGGACAGC GTGGTTTGGCAGCCATCTTC	179
GR3000_106	*Mrpl48*	TGGACAGAGCTGTGCTTTGG CACAGCAGCGCTGAGGTTTA	151
Gene	**Full name**	**Forward & reverse (5'>3')**	**Product length (bp)**
Actb	Beta-actin	TGAACCCTAAGGCCAACCGTG ACACAGCCTGGATGGCTACG	90
Atoh1	Atonal bHLH transcription factor 1	TCTGACGAGGCCAGTTAGGA TCCGAAGTCACATCGTTGCT	156
Neurod1	Neuronal differentiation 1	AGGTGGTACCCTGCTACTCT GCTGGGACAAACCTTTGCAG	159
Neurod2	Neuronal differentiation 2	TAAGGGGCTGCTGAGTTTCG GGAGATTCGTGTTGGGGTGA	160
Neurod6	Neuronal differentiation 6	AGAGGCTCCAGGAGACGATG TGGGATTCGGGCATTACGAC	155

Supplemental Table 3. Lists of MR, overlapping and GR binding sites. Available on https://doi.org/10.1210/en.2016-1422

Supplemental Table 4. Lists of MR, overlapping and GR target genes. In the MR-exclusive list, 35 of the MR300 binding sites have an overlapping MR3000 peak, which is listed under 'corresponding binding site'. Available on https://doi.org/10.1210/en.2016-1422

Supplemental Table 5. Gene ontology for MR, overlapping and GR target genes. The top 10 functional annotation clusters with an enrichment score (ES) above 1.3. The ES is the negative logarithm of the geometric mean of p-values from all terms within the cluster. BP = biological process, CC = cellular component, MF = molecular function

MR-exclusive target genes

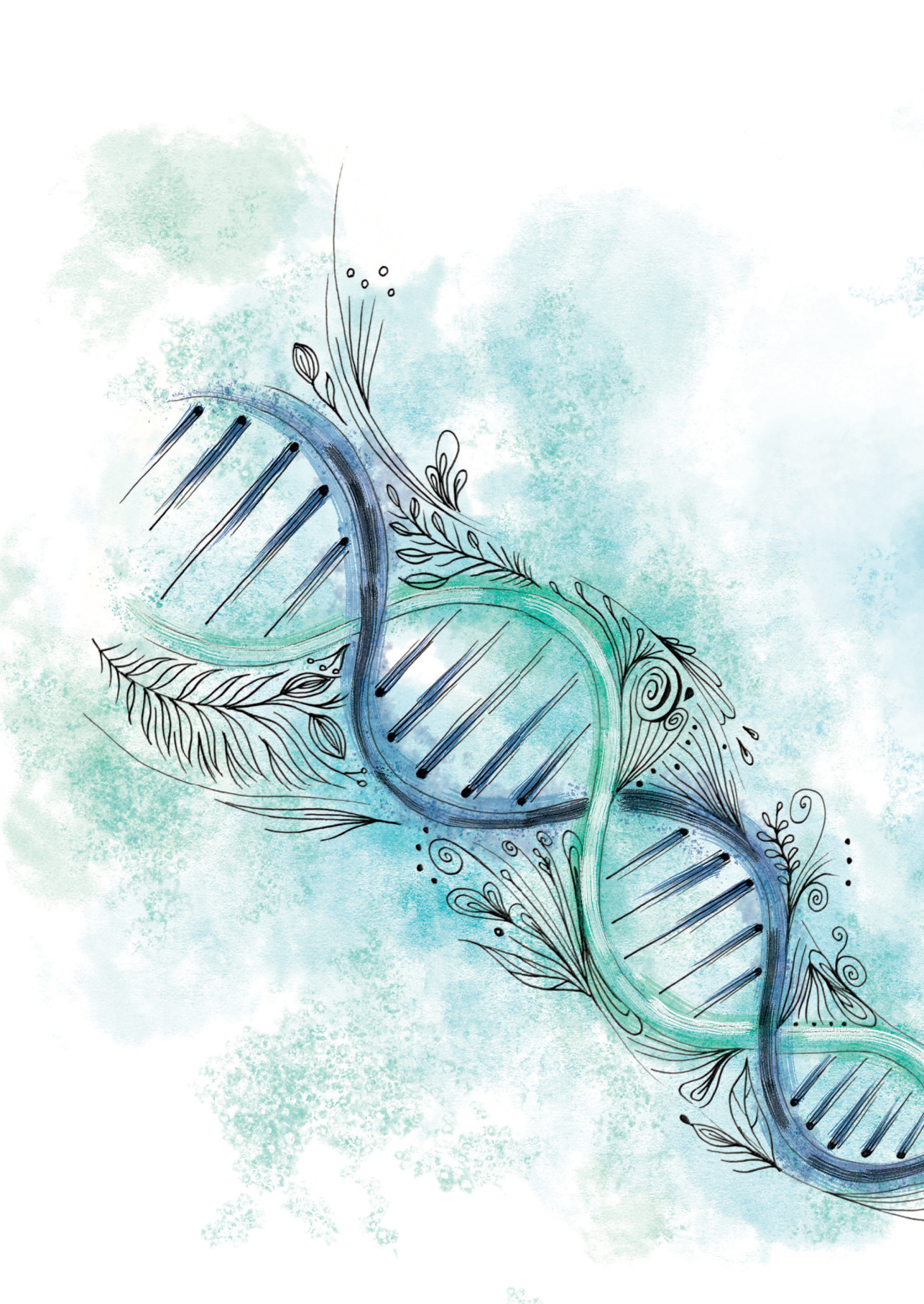
GO term(s)	Category	Enrichment score
Nucleotide binding	MF	3.50
Ion transport, voltage-gated channel activity	MF	2.43
Sodium channel activity	MF	2.21
Ion homeostasis	BP	1.95
Immunoglobulin, cell adhesion	BP	1.78
Regulation of cell projection assembly	BP	1.72
Membrane/insoluble fraction	CC	1.69
Enzyme/kinase binding	MF	1.63
Endoplasmic reticulum	CC	1.53
Calcium ion transport/signaling	BP	1.51

MR-GR overlapping target genes

GO term(s)	Category	Enrichment score
Cytoskeleton, microtubule	CC	1.79
Synapse	CC	1.76
Positive regulation of protein binding	BP	1.52
Membrane/insoluble fraction	CC	1.49
Ion binding	MF	1.41
Regulation of synaptic plasticity/transmission	BP	1.40

GR-exclusive target genes

GO term(s)	Category	Enrichment score
Cell/neuron projection, dendrite	CC	6.48
Enzyme/kinase binding	MF	3.30
Membrane/insoluble fraction	CC	3.26
Cell adhesion	BP	2.87
Apoptosis	BP	2.52
Response to endogenous/hormone stimulus	BP	2.39
Response to oxidative stress	BP	2.30
Synaptic vesicle	CC	2.19
Cytoskeleton organization	BP	1.78
Immunoglobulin	CC	1.76



CHAPTER 3

Identification of mineralocorticoid receptor target genes in the mouse hippocampus

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Abstract

Brain mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) respond to the same glucocorticoid hormones, but can have differential effects on cellular function. Several lines of evidence suggest that MR-specific target genes must exist, and might underlie distinct effects of the receptors. Our goal was to identify MR-specific target genes in the hippocampus, a brain region where MR and GR are co-localized and play a role in the stress response. Using genome-wide binding of both receptor types, we previously identified MR-specific, MR-GR overlapping and GR-specific putative target genes. We now report altered gene expression levels of such genes in the hippocampus of forebrain MR knockout (fbMRKO) mice, sacrificed at the time of their endogenous corticosterone peak. Of those genes associated with MR-specific binding, the most robust effect was a 50% reduction in *Jun dimerization protein 2* (*Jdp2*) mRNA levels in fbMRKO mice. Downregulation was also observed for the MR-specific *Nitric oxide synthase 1 adaptor protein* (*Nos1ap*) and *Suv3 like RNA helicase* (*Supv3l1*). Interestingly, the classical glucocorticoid target gene *FK506 binding protein 5* (*Fkbp5*), that is associated with MR and GR chromatin binding, was expressed at substantially lower levels in fbMRKO mice. Subsequently hippocampal *Jdp2* was confirmed to be upregulated in a restraint stress model, posing *Jdp2* as a bona fide MR target that is also responsive in an acute stress condition. Thus, we show that MR-selective DNA binding can reveal functional regulation of genes, and further elucidates distinct MR-specific effector pathways.

Introduction

Endogenous glucocorticoid hormones affect brain function via two closely related nuclear receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The ligand concentration in part determines the specific MR/GR responses. High affinity MRs are occupied by endogenous corticosteroids at basal conditions, and have been found to be more relevant in the initial phase of a stress response (1, 2). In contrast, the lower affinity GRs get activated only at higher glucocorticoid levels, around the peak of the circadian rhythm and during a stress response. While GRs are expressed widely throughout the central nervous system, brain glucocorticoid binding MRs are mainly restricted to limbic areas (3).

In the hippocampus MR and GR are crucial for spatial memory and the modulation of cognition, mood and behavior (3). Within the CA1 hippocampal subregion, MR and GR mediate opposite glucocorticoid effects on pyramidal neuron excitability (4), via transcriptional mechanisms (5). Also spatial learning in rodents is differentially affected by MR and GR signaling, with MR modulating response selection and GR being essential for memory consolidation (6, 7). Because of intrinsic MR-mediated effects that oppose those of GR, it has long been argued that MR-specific target genes must exist (8). The existence of MR-specific transcriptional coregulators (9, 10) also argues this point. However, many effects that can be attributed specifically to MR function so far are rapid non-genomic effects, mediated by the membrane variant of the receptor (11, 12).

Several classical genomic MR-targets have been described in various tissues over the past two decades, such as *FK506 binding protein 5* (*Fkbp5*) (13), *glucocorticoid-induced leucine zipper* (*Gilz*) (14), *period circadian clock 1* (*Per1*) (15) and *serum/glucocorticoid regulated kinase 1* (*Sgk1*) (16). However, these genes are all known to be also GR responsive (17-20). Of note, the two receptors can bind their target DNA as homodimers, but also heterodimerization of MR/GR has been described (15). While MR-selective transrepression and transactivation may occur (21, 22), to date, no hippocampal genomic targets have been reported that are strictly MR-dependent. Transcriptional changes have been attributed to MR function (23), but were not formally proven to be direct targets of the receptor and might thus be affected by MR activity in an indirect manner. However, while Glucocorticoid Response Element (GRE) presence seems crucial for both MR and GR DNA binding in the hippocampus, binding sites for NeuroD transcription factors were found selectively at MR-bound loci (24). NeuroD factors could coactivate glucocorticoid-induced

transactivation and were indeed present near MR-specific binding sites, suggesting that specific GRE-dependent MR target genes do exist.

The current study assessed if direct MR binding to the hippocampal DNA led to expression regulation of the nearby gene. Based on our recent work that defined MR-specific, MR-GR overlapping and GR-specific chromatin binding sites and corresponding putative target genes within the rat hippocampus (24), we examined mRNA levels of several genes in each of these categories. Forebrain MR knockout (fbMRKO) mice showed altered expression for a subset of genes, including downregulation of the mixed MR/GR target *Fkbp5*, and the MR-specific *Jun dimerization protein 2* (*Jdp2*), *Nitric oxide synthase 1 adaptor protein* (*Nos1ap*) and *Suv3 like RNA helicase* (*Supv3l1*) mRNA levels. Subsequently, corticosterone responsiveness of *Jdp2*, one of the genes having an MR-bound promoter, was validated in mice that were exposed to different durations of restraint stress.

Material and methods

Animals

Male homozygous forebrain-specific MR knockout (fbMRKO) and control c57bl/6 mice (n=7) aged 8-9 weeks, were housed on a 12-hour light/12-hour dark reversed cycle (lights off at 9:00AM). The fbMRKO mice were generated using MR^{flox} mice, having MR exon 3 flanked by loxP sites, and mice expressing Cre recombinase controlled by the CAMKIIα gene (25). Male $MR^{flox/flox}CamKCre^{Cre/wt}$ mice were crossed with female $MR^{flox/flox}$ mice to generate fbMRKO ($MR^{flox/flox\_Cre}$) and control ($MR^{flox/flox\_wt}$) offspring. As the breeding unexpectedly generated more fbMRKO than control mice, only part of the control animals were littermates. No differences were found in expression levels between littermate and non-littermate controls in any of the genes measured. Mice were transferred to a novel cage 20 min before harvesting the tissue, and sacrificed around the time of their endogenous corticosterone peak, between 9:30AM-12:00PM. We assessed the expression of MR, overlapping and GR putative target genes in this condition, as both receptor types are activated at peak of the diurnal corticosterone rhythm. The novel cage was included in the protocol to ensure MR and GR binding for ChIP analysis in the same animals, under the assumption that mRNA levels will not be affected in this short time span. From all mice trunk blood was collected, and hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

For validation of *Jdp2* downregulation, male fbMRKO (n=14) and littermate controls (n=10) aged 8-12 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 7:00AM). Mice were bred as described above. Sacrifice took place under baseline conditions, between 9:30-10:30AM. Brains were collected and snap-frozen in liquid nitrogen for later analysis.

For MR binding site validation in the mouse brain, male c57bl/6 mice (n=5) aged 16-19 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 8:00AM), and were sacrificed in the afternoon 60 min after an IP injection of 3.0 mg/kg corticosterone (Sigma) dissolved in 5% ethanol in saline, ensuring MR binding. Hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

Male Balb/c mice (n=3-6) aged 8-15 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 6:00AM), and were exposed to various periods of restraint stress (0-30-60-120-240 min) and sacrificed directly afterwards, between 9:30AM-2:00PM. At this time of the diurnal corticosterone trough, both MR and GR DNA binding can be enhanced in response to stress (15) and consequential gene expression changes compared to non-stressed control mice could be revealed. From all mice trunk blood was collected, and hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

All experiments were performed according to the European Commission Council Directive 2010/63/EU and the Dutch law on animal experiments and approved by the animal ethical committee from Utrecht University, University of Amsterdam, or the German Regierungspräsidium Tübingen.

Plasma measurements

Trunk blood was centrifuged for 10 min at 7000x*g*, after which plasma was transferred to new tubes. Corticosterone levels of the fbMRKO experiment were determined using an Enzyme ImmunoAssay (EIA, Immunodiagnostic Systems), and ACTH and corticosterone levels of the restraint stress mice were determined using an Enzyme-Linked ImmunoSorbent Assay (ELISA, IBL International), according to the manufacturers' instructions.

Target gene selection

MR-specific, MR-GR overlapping and GR-specific binding sites were annotated to the nearest gene (24). In order to increase the chances of correct annotation and identifying functional target genes, we focused on binding sites located intragenic or in the proximal promoter (up to -5 kb). Furthermore, hippocampal expression (26) of the putative target genes, the degree of coexpression with NeuroD factors (Neurod1/2/6) and face validity of chromatin immunoprecipitation-sequencing (ChIP-seq) peaks were assessed. The total numbers of putative target genes measured for MR-specific, overlapping (including classical targets), and GR-specific subset were 12, 10 and 9 respectively.

Table 1. Primer sequences used for qPCR on mouse hippocampal ChIP samples. See Table 3 for binding site details.

Binding site	Nearest gene	Forward & reverse (5'>3')	Product length (bp)
GR3000_1726	*Acsl6*	CCTGCCAGGAGAGCAGATG TGTGCAGGAAGGCAAGTTCT	178
MR3000_740 GR3000_34	*Fkbp5*	TGCCAGCCACATTCAGAACA TCAAGTGAGTCTGGTCACTGC	122
MR3000_1054	*Jdp2*	AAGTAAGACCGCGACCTACA AAATACCCAGTGCAGAGACGAA	192
MR300_473 GR3000_599	*Kif1c*	GCTGGGGTGTACACAGATGG TGACTAGCCAGAGCAGTATGTC	156
GR3000_106	*Mrpl48*	AGCTGTGCTTTGGAAGCCTA CATAAGGTGGGCCACACTCC	170
MR300_196	*Nos1ap*	CCTCCGATGCTGCTTGGATA CAGACCGAGCCAGCGATAAG	197
MR3000_738 GR3000_12	*Per1*	GGAGGCGCCAAGGCTGAGTG CGGCCAGCGCACTAGGGAAC	73
MR300_503	*Rilpl1*	CAGGCAGATGCCAGGCT CCCATGCCTGTTCCTCTAGT	106
MR3000_359	*Supv3l1*	TGCAGGGATTCGATGGACAG CTCTGAGCCACCTCTCAAGC	165
MR3000_641 GR3000_1603	*Zfp219*	AGTCCATCACATTCTGTTGCTTTC TAGTCAGCTATGACCATGCAGT	131

ChIP-qPCR

For MR binding validation in the mouse, we performed ChIP-qPCR on hippocampal tissue of wild type mice (n=5) as described previously (27). Hippocampal hemispheres were cryosectioned at 30 µm before crosslinking with 2 mM disuccinimidyl glutarate, followed by 1% formaldehyde. Fixated tissue was suspended, nuclei were isolated and sonicated

for 10 rounds (30 seconds ON/30 seconds OFF) using a Biorupter Pico (Diagenode). Chromatin of two hemispheres of the same animal were pooled and used for a single ChIP sample (500 µL) to measure MR binding with 5 µg of anti-MR antibody (21854-1-AP, ProteinTech). Immunoprecipitation was performed with 50 µL magnetic Protein A beads (Dynabeads™, Invitrogen). Background signal was detected for each sample with a sequential ChIP using 5 µg of control IgG antibody (ab37415, Abcam). Pellets were dissolved in 50µL 10mM Tris-HCl pH 8. Subsequently, qPCR was performed on 5x diluted ChIP samples, with primers that were designed to span the GRE of the MR binding sites and are listed in **Table 1**.

Real-time quantitative PCR

Mouse hippocampal hemispheres were homogenized in TriPure (Roche) by shaking the tissue with 1.0-mm-diameter glass beads for 20 seconds at 6.5 m/s in a FastPrep-24 5G instrument (MP Biomedicals). Total RNA was isolated, cDNA was generated and RT-qPCR was performed as described previously (24). As *Actb* (beta-actin) expression was regulated between fbMRKO and control mice, genes of interest were normalized against the in both experiments stably expressed housekeeping gene *Rplp0*, encoding a ribosomal protein. Primer sequences are listed in **Table 2**.

In situ hybridization

Frozen brains were sectioned at 18 µm in a cryostat microtome, collected on Super Frost Plus slides, and stored at -80°C until further use. *In situ* hybridization using ^{35}S UTP-labeled ribonucleotide probes for *Jdp2* was performed as described previously (28).

Table 2. Primer sequences used for RT-qPCR on mouse hippocampus.

Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
Acsl6	Acyl-CoA synthetase long-chain family member 6	TCTCAGGGAATGGACCCTGT CCTCTTGGTAGGACAGCCAC	135
Bhlhb9	Basic helix-loop-helix domain containing, class B9	AACTCACCTGGCCAGCAATC CTCTGGCTGCCTTGGGATTT	187
C4ST1 (Chst11)	Chondroitin 4-sulfotransferase 1	GAATTTGCCGGATGGTGCTG AGCAGATGTCCACACCGAAG	117
Camk1d	Calcium/calmodulin-dependent protein kinase ID	GCATCGAGAACGAGATTGCC CCAGACACAAGTTGCATGACC	114
Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2	AGAACTGCACACTGGTCGAG ACCAGGATCACAGTTGCCAG	85

Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
Fkbp5	FK506 binding protein 5	TCCTGGGAGATGGACACCAA TTCCCGTACTGAATCACGGC	113
Gilz (Tsc22d3)	Glucocorticoid-induced leucine zipper	TGGCCCTAGACAACAAGATTGAGC CCACCTCCTCTCTCACAGCAT	78
Hsd17b11	Hydroxysteroid (17-beta) dehydrogenase 11	CGCAGGACCCTCAGATTGAA GGAGCAGTAAGCCAGCAAGA	167
Jdp2	Jun dimerization protein 2	TACGCTGACATCCGCAACAT CGTCTAGCTCACTCTTCACGG	100
Kif1c	Kinesin family member 1C	TTAATGCCCGTGAGACCAGC AAGCTTTTGGGGGCATCCTT	106
Mrpl48	Mitochondrial ribosomal protein L48	CAGTATGTCCACCGCCTCTG CTCGCTCATGGGTGGTAAGG	145
Nos1ap	Nitric oxide synthase 1 adaptor protein	TGGAATTCAGCCGAGGTGTG GGAAGGGAGCAGCATTCGAG	131
Nr3c1 (GR)	Nuclear receptor subfamily 3, group C, member 1	CCCTCCCATCTAACCATCCT ACATAAGCGCCACCTTTCTG	89
Nr3c2 (MR)	Nuclear receptor subfamily 3, group C, member 2	TCCAAGATCTGCTTGGTGTG CCCAGCTTCTTTGACTTTCG	239
Per1	Period circadian clock 1	ACGGCCAGGTGTCGTGATTA CCCTTCTAGGGGACCACTCA	162
Rilpl1	Rab interacting lysosomal protein-like 1	ACGAGCTCAAGTCCAAGGTG AGTCGCTTGATCCCCGATTC	148
Rplp0	Ribosomal protein, large, P0	GGACCCGAGAAGACCTCCTT GCACATCACTCAGAATTTCAATGG	85
Sgk1	Serum/glucocorticoid regulated kinase 1	AGAGGCTGGGTGCCAAGGAT CACTGGGCCCGCTCACATTT	129
Supv3l1	Suv3 like RNA helicase	CTCACTCGGCCTCTAGACAAG TCCACGTCCAGAGAATGGGA	170
Zfp219	Zinc finger protein 219	GATCTGCAGCGCTACTCCAA TGCACGAGTCTCAGACCAAC	96

Statistics

In the fbMRKO experiment, independent *t*-tests were used, taking $P<0.01$ as significance cut-off to correct for multiple gene testing. For the ChIP-qPCR validation we performed one-tailed paired *t*-tests. The predictable directionality, i.e. MR signal is higher than background IgG signal, justifies the use of a one-tailed test. As one may argue that a decrease in signal would also be relevant, we note that significant *P*-values were all <0.025, and therefore would also be significant using a two-tailed test. We considered a paired test appropriate as MR and IgG are measured on the same chromatin sample, and this allows correction for the corresponding background levels. Again, one-tailed unpaired *t*-tests gave essentially the same results. For one of the genes, *Nos1ap*, one of the samples

was excluded from analysis because of a missing value due to non-detectable IgG levels. For the time course of restraint stress, a one-way ANOVA was performed with Holm-Sidak's multiple comparison post-hoc tests. In the *in situ* measurements of the fbMRKO animals, unpaired *t*-tests were performed. Results were considered significantly different when $P<0.05$ unless stated otherwise. GraphPad Prism 7 was used to analyze the data. All graphs show individual values and data are further depicted with means ± SEM.

Table 3. Selected putative target genes to validate.

Binding site	GRE sequence (rat/mouse)	Annotation	Distance from TSS (bp)	Associated gene	ABA hippocampal expression
MR300_225	AGAACATTATGTTCC AAAACATCAGGATCC	Intron	116761	*Camkk2*	10.34
MR3000_360	GGAACACTCTCTTCC GGAACTCTCTCTTCC	Intergenic	-1071	*Hsd17b11*	3.98
MR3000_1054	AGAGCTCTTTGTGTT AGAATTCTTTGTGTT	Intergenic	-3983	*Jdp2*	13.58
MR300_196	CTCACACTTTCTCCC CTAGCACTCTCTCCC	Intron	233500	*Nos1ap*	11.50
MR300_503	CAACCTCTTTCTTCC CAACCCTCTTTCTCC	Intron	12715	*Rilpl1*	15.52
MR3000_359	TGTGCTTTCTGTTCC GGTGCTTTTTGTTAC	Intron	1661	*Supv3l1*	0.83
MR300_713 GR3000_248	AGAGCAGGCTGTTCT AAAACAGCCTGGTCT	Intron	95108	*Camk1d*	2.64 (mainly CA)
MR3000_740 GR3000_34	AGAACAGGGTGTTCT AGAACAGGGTGTTCT	Intron	62931	*Fkbp5*	8.86
MR300_473 GR3000_599	GGGACTGGAAGTTCC GGAACTTCCAGTCCC	Intron	9921	*Kif1c*	2.94
MR3000_738 GR3000_12	GGAACATCGTGTTCT GGAACATCGTGTTCT	Intergenic	-3357	*Per1*	3.06
MR3000_641 GR3000_1603	ACACCAGGATGTTCC ACACCAGGATGTTCC	Intergenic	-2125	*Zfp219*	2.62
GR3000_1726	TGAACTTGCAGCGTT TGAGCTTGCAGCATT	Intergenic	-1931	*Acsl6*	15.52
GR3000_647	AGGACTGTTAGTACT AGGGCTTTAGTACT	Intergenic	-3526	*Bhlhb9*	9.05
GR3000_193	AGAACTGTCTGCACC AGAACTCTCCATCAG	Intron	121265	*C4ST1*	7.28
GR3000_106	GGCTCTCCTTGTGCT GGCTCTCCTTGTGCC	Intron	24445	*Mrpl48*	4.71

Additional binding site information can be found in supplemental data of (24). Sequences represent the rat GRE (upper) and mouse GRE (lower) with mismatches to the rat sequence in red. GRE = glucocorticoid response element, TSS = transcription start site, ABA = Allen Brain Atlas

Results

In order to explore the functional effects of previously detected MR/GR DNA binding, i.e. transcription regulation, binding sites were associated to their nearest gene. This resulted in lists of MR-specific, MR-GR overlapping and GR-specific putative target genes (24). Gene expression levels, for a subset of each category (**Table 3**), were measured in forebrain-specific MR knockout (fbMRKO) mice at the time of their diurnal corticosterone peak. MR mRNA expression was indeed abolished, and GR mRNA was slightly upregulated in the hippocampus of fbMRKO mice (**Figure 1A**), confirming earlier reports (25). MR protein levels also showed efficient knockdown (29). Furthermore, no differences were found in plasma corticosterone levels of these animals at the time of sacrifice (**Figure 1B**). As the studied target loci were originally detected in the rat brain (24), we validated MR binding in mice. ChIP-qPCR confirmed hippocampal MR binding at the *Jdp2* ($P = 0.0124$), *Kif1c* ($P = 0.0087$), *Nos1ap* ($P = 0.0172$), *Rilpl1* ($P = 0.0098$), and *Zfp219* ($P = 0.0049$) loci in wild type (WT) mice, while this signal did not exceed background IgG levels at the GR-specific sites near *Acsl6* ($P = 0.4410$) and *Mrpl48* ($P = 0.2142$) (**Figure 1C**). Only for *Supv3l1* ($P = 0.1784$) we were unable to detect the expected MR binding. Also for classical target genes *Fkbp5* ($P = 0.0246$) and *Per1* ($P = 0.0066$) an MR enrichment was demonstrated.

Several MR-specific putative targets showed lower expression levels in the fbMRKO compared to WT mice (**Figure 2A**). The most robust effect was found in the *Jdp2* mRNA levels, which were reduced by 50% ($P < 0.0001$). Other differentially expressed genes were MR-specific *Nos1ap* ($P = 0.0005$) and *Supv3l1* ($P = 0.0061$), and MR-GR overlapping *Camk1d* ($P = 0.0016$) and *Kif1c* ($P = 0.0022$), which were also all downregulated in the fbMRKO compared to WT mice (**Figure 2A, 2B**). Moreover, two of the GR-specific genes, *Acsl6* ($P = 0.0002$) and *Mrpl48* ($P = 0.0065$) were expressed at lower levels, and *C4ST1* showed a trend of lowered expression ($P = 0.0138$) (**Figure 2C**).

Besides the brain-related putative MR/GR target genes, we measured the expression of the classical target genes *Fkbp5*, *Gilz*, *Per1* and *Sgk1* (**Figure 2D**). These genes are all known to be bound and/or regulated by both MR and GR; our identified MR-GR overlapping target subset contained *Fkbp5* and *Per1* (**Table 3**). Of the four classical targets only *Fkbp5* was downregulated in fbMRKO mice, to 44% of the levels observed in WT animals ($P < 0.0001$).

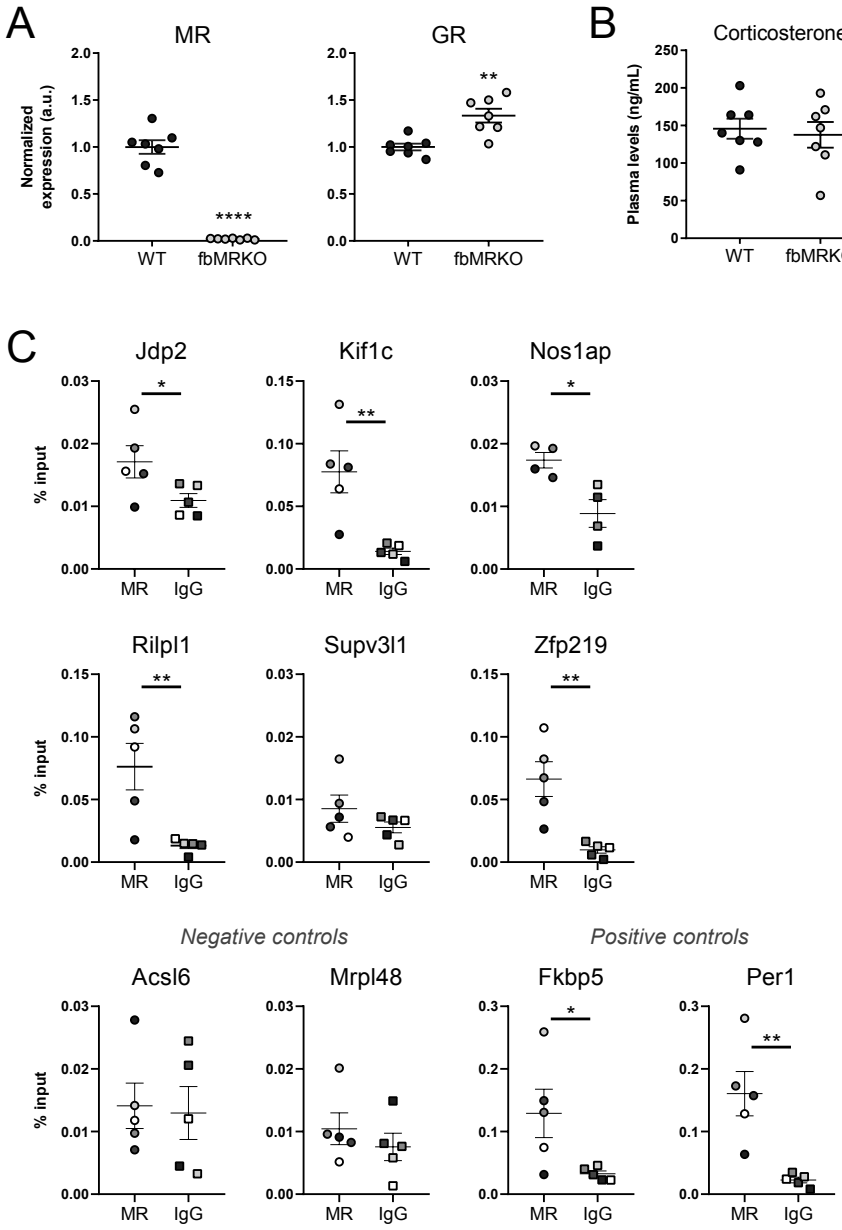

Figure 1. Validation of MR detection in wild type (WT) mice and absence of MR in forebrain MR knockout (fbMRKO) mice. **A)** Hippocampal mRNA levels showing MR downregulation and slight GR upregulation, and **B)** unaltered plasma corticosterone levels in fbMRKO versus wild type (WT) mice; assessed by independent *t*-tests. **C)** MR binding assessed by ChIP-qPCR in the hippocampus of WT mice, along with an IgG background signal per sample; assessed by one-tailed paired *t*-tests. Corresponding measurements are depicted in the same color. GR-specific targets *Acsl6* and *Mrpl48* served as negative controls; classical glucocorticoid targets *Fkbp5* and *Per1* served as positive controls. a.u. = arbitrary unit, * $P<0.05$, ** $P<0.01$, **** $P<0.0001$

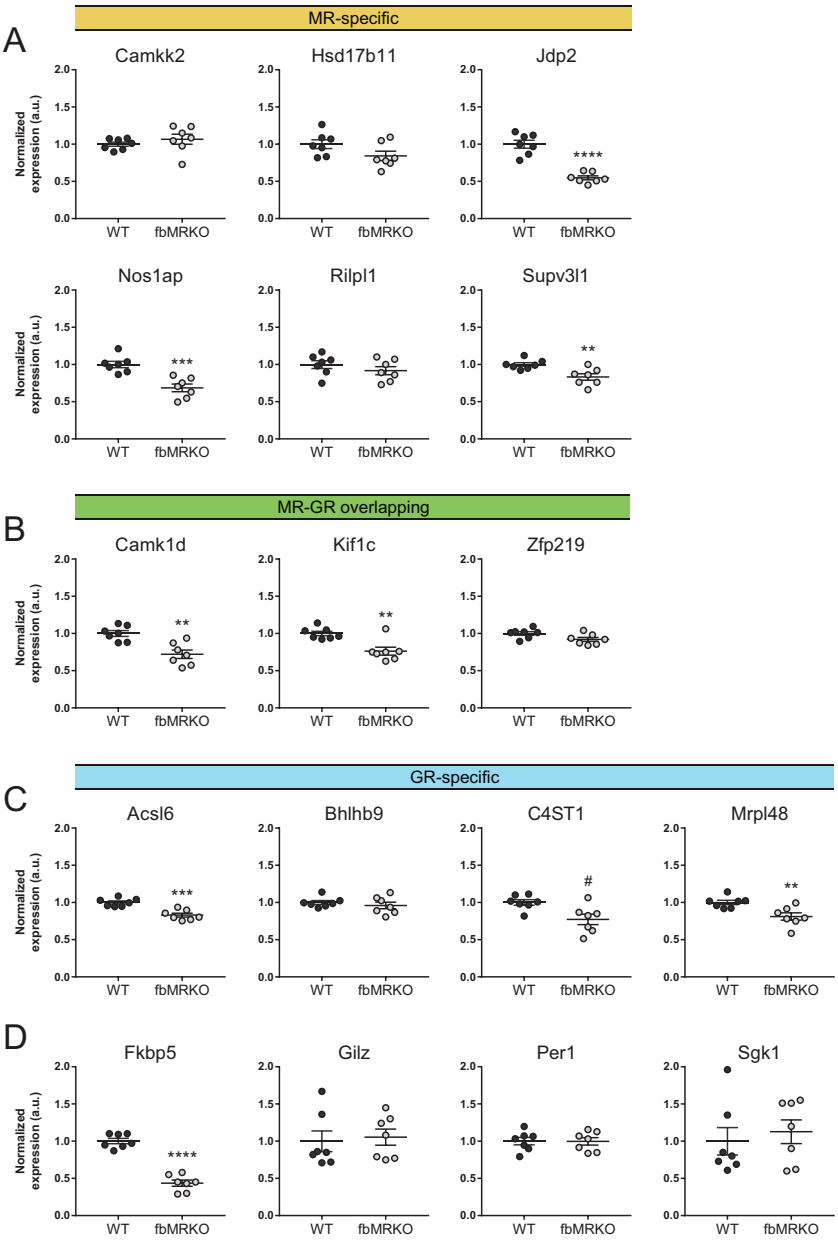

Figure 2. Hippocampal mRNA levels of glucocorticoid target genes assessed in wild type (WT) and forebrain MR knockout (fbMRKO) mice. Gene expression of **A)** MR-specific, **B)** overlapping and **C)** GR-specific targets and **D)** classical glucocorticoid targets in fbMRKO versus WT mice; assessed by independent *t*-tests with $P<0.01$ as significance cut-off. Other genes measured, but not differentially expressed between WT and fbMRKO mice: *Adam23*, *Arl8b*, *Dgkb*, *Els1*, *Myo16* and *Nob1* as MR-specific targets; *Grb2*, *Luzp1* and *Map1lc3b* as overlapping targets; *Arntl*, *B3galt1*, *Map2k5*, *Pglyrp1* and *Slc3a2* as GR-specific targets. a.u. = arbitrary unit, # $P<0.05$ (considered a trend), ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$

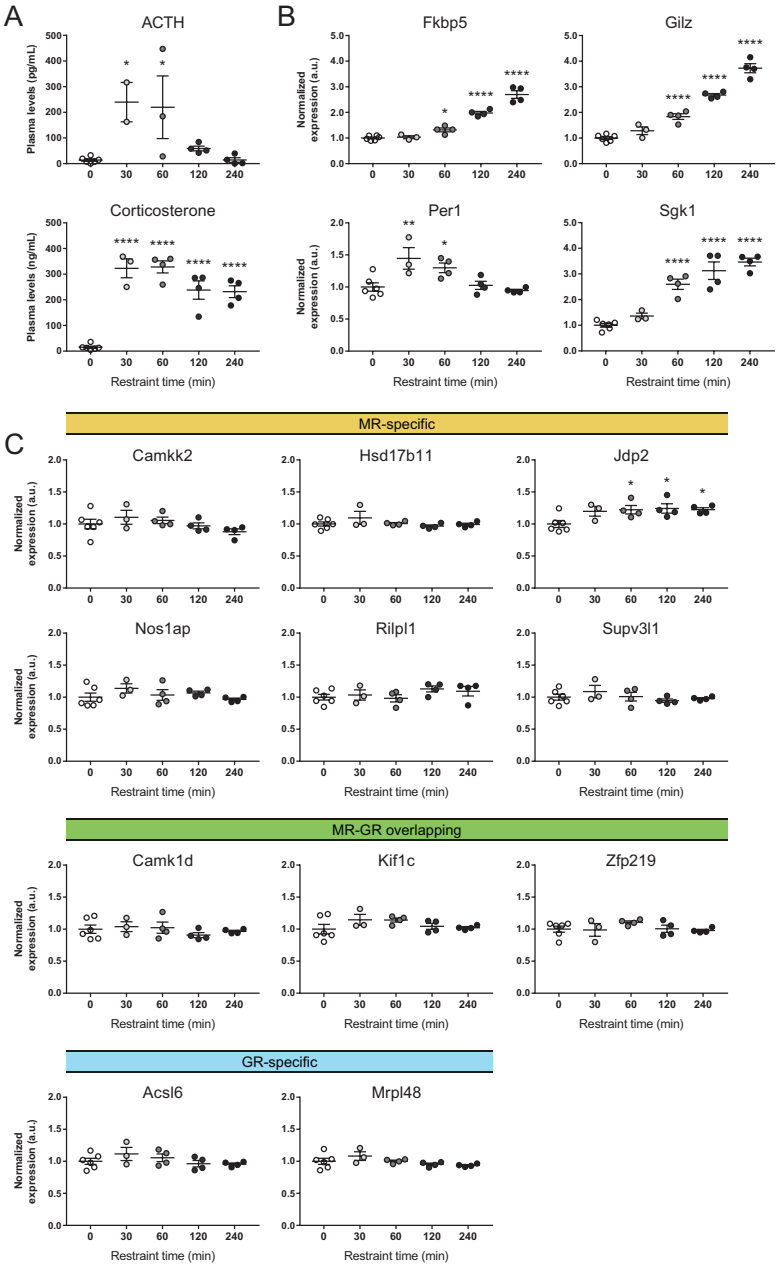

Figure 3. Hippocampal mRNA levels of glucocorticoid target genes assessed in a restraint stress model. **A)** Plasma ACTH and corticosterone levels after different durations of restraint stress. **B)** Validation of time-dependent classical glucocorticoid target gene activation upon restraint stress. **C)** Gene expression of MR-specific, overlapping and GR-specific targets after different durations of restraint stress. All assessed by one-way ANOVA with Holm-Sidak's post-hoc tests. ACTH = adrenocorticotropic hormone, a.u. = arbitrary unit, * $P<0.05$, ** $P<0.01$, **** $P<0.0001$

Next, we aimed to show regulation of the target genes in an acute stress context. Even though MR is substantially occupied by ligand under basal glucocorticoid levels, MR (and GR) DNA binding and subsequent transcriptional effects can be enhanced by a rise of corticosterone (15). Hippocampal gene expression was assessed in mice that were exposed to restraint stress of different durations (0-30-60-120-240 min). Plasma corticosterone levels were increased after all durations of restraint stress, but tend to return to baseline at 120 min and 240 min, in line with the fact that ACTH levels were normalized at these time points (**Figure 3A**).

Of the classical glucocorticoid target genes, *Fkbp5*, *Gilz* and *Sgk1* were upregulated after 60, 120 and 240 min of restraint (**Figure 3B**). *Per1* showed a transient increase, with elevated levels at 30 min and 60 min, which had declined again from 120 min restraint stress. Interestingly, the MR-exclusive target gene *Jdp2* that was mostly affected in the fbMRKO mice showed an increase in response to stress (**Figure 3C**), in animals that were exposed to restraint for 60 to 240 min. Other genes associated with MR and/or GR binding loci that we had selected for validation did not show transcriptional effects upon restraint stress (**Figure 3C**).

Finally, we confirmed *Jdp2* downregulation measured by *in situ* hybridization in an independent experiment in fbMRKO (**Figure 4**). In absence of MR, *Jdp2* mRNA levels were decreased in the principal neurons of the dorsal hippocampus, as apparent from significant lower expression in the CA2 ($P = 0.0001$), CA3 ($P = 0.0357$) and dentate gyrus ($P = 0.0005$) subregions. For the CA1 this occurred at the trend level ($P = 0.0901$).

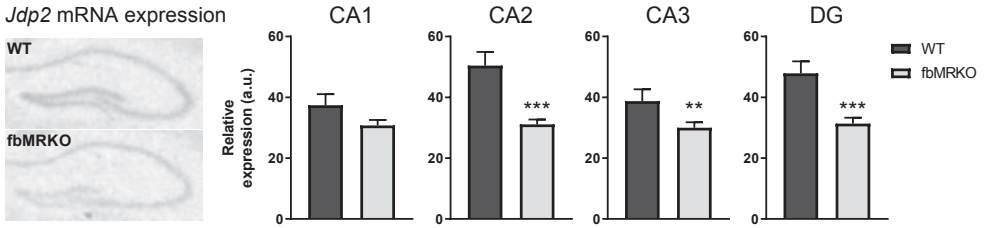

Figure 4. Validation of hippocampal *Jdp2* downregulation in forebrain MR knockout (fbMRKO) mice compared to wild type (WT) mice, detected by *in situ* hybridization; assessed by unpaired *t*-tests. On the left is depicted a representative scanned autoradiograph film per genotype. Gene expression is quantified per subregion of the hippocampus: cornu ammonis (CA)1, CA2, CA3 and the dentate gyrus (DG). a.u. = arbitrary unit, ** $P<0.01$, *** $P<0.001$

Discussion

Based on non-overlapping MR-GR binding sites, we defined putative MR-specific and GR-specific hippocampal target genes. We identified *Jdp2* as a likely MR-specific transcriptional target, that is both downregulated in fbMRKO mice and upregulated in response to restraint stress. Also *Nos1ap* and *Supv3l1*, two other genes linked to MR-specific binding sites, were expressed at a lower levels in fbMRKO mice, but did not change upon restraint stress. Classical glucocorticoid target genes *Fkbp5*, *Gilz*, *Per1* and *Sgk1* all responded to restraint stress by increased transcription. Of these targets, only *Fkbp5* showed a substantially lower hippocampal expression in the absence of MR.

Both technical and biological factors could explain the limited success in validating MR-specific genomic targets. The annotation of binding sites to the nearest gene is not without error, as it is possible that another neighboring gene is affected by the binding locus assessed. We do not have data on spatial chromatin organization or RNA polymerase activity in the same experimental setup, which could enable the proper linking of binding loci to the actual site of transcriptional activity (30). To lower the chance of false positive annotations, we did focus on binding sites that were located within genes or (proximal) promoter regions. However, even in the case that the putative target is legitimate, we might still have false negative results on gene expression changes. Because the hippocampus consists of several subregions and various cell types, we could be unable to detect MR-dependent regulation that is constrained to a subset of hippocampal cells. While the ChIP-seq signal can be strong enough to withstand dilution, gene regulation might be diluted when the average gene expression over the whole hippocampus is assessed, as fold change in hippocampal mRNA expression tends to be modest in response to steroids (31). Despite possible false negative results, we were able to find robust changes in several MR-specific and classical glucocorticoid target genes.

It is of note that gene regulation by MR knockout and restraint stress was validated in a mouse model, while the MR/GR binding loci were obtained from experiments in rats. We were able to show MR binding in the mouse hippocampus at five out of six MR targets originally detected in the rat brain. Evolutionary conservation can increase the predictive value of functional GREs (32, 33). Moreover, as brain MR/GR-mediated regulation is considered part of a general adaptive response, one would expect genes regulated in rat to also be affected in mice. However, the species difference is an additional potential cause for absence of mRNA regulation.

The fbMRKO animals become MR deficient during embryonic development, and loss of MR protein is completed after birth (25). In our experiment downregulated MR expression was validated, and slight upregulation of GR expression in the hippocampus was observed as described before (25). It is possible that MR-dependent gene expression is normalized due to compensation by GR or other factors. We cannot exclude that such compensatory mechanisms might as well affect expression of *Jdp2*, *Nos1ap* and *Supv3l1* in the fbMRKO mice. Also, redundancy in gene regulation is not uncommon, and while complete dependence of target genes to a single transcription factor can happen (34), it is rare in case of MR and GR signaling. In addition, binding of nuclear receptors such as MR can have permissive effects on chromatin, and could be necessary but not sufficient for transcription. In fact, as little as 13% of GR binding sites can be linked to transcriptional activity (35). Thus, the lack of transcriptional effects might reflect a context dependency.

To start looking at MR regulation in a relevant context, we chose a restraint stress paradigm in wild type mice as a more physiological setting. Mice were stressed in the morning, to make sure that basal corticosterone levels were low, and MR activation not necessarily fully maximal (36). The classical glucocorticoid target genes all responded in this acute stress situation, and of the MR-specific targets identified in the fbMRKO mice only *Jdp2* expression was affected. Non-regulated genes in the restraint stress experiment might still be MR-dependent, but at a lower EC50 (37), or in different contexts, like in behavioral paradigms in which fbMRKO animals show changed phenotypes, such as working memory in a radial maze (25).

For the genes associated with GR-specific chromatin binding, *Acsl6* and *Mrpl48* showed lower expression levels in the fbMRKO mice. In general, the effect size on specifically GR-associated target gene expression was less pronounced. The fact that these GR targets are downregulated, while expression of GR itself is slightly upregulated in fbMRKO mice seems contradictory. However, this could be a result of indirect effects of MR deficiency. Another explanation is that GR binding takes place at a negative GRE, where GR leads to repression (instead of activation) of the nearby gene (38, 39).

More interestingly, several overlapping targets were downregulated in fbMRKO mice: the newly identified *Camk1d* and *Kif1c*, and the classical target *Fkbp5*. This suggests that MR is needed for expression of these genes in the hippocampus. The GR compensatory upregulation does not seem to prevent dysregulation of these combined target genes in the absence of MR. It is likely that heterodimerization of MR and GR is involved in the regulation of overlapping binding sites. *Fkbp5* expression was recently shown to

be modulated by MR-GR heterodimers (15). The observation that *Fkbp5* expression is lowered in fbMRKO mice, can represent functional consequences of the absence of one of the heterodimerization partners. Fkbp5 is part of an ultra-short feedback loop, where it is induced by glucocorticoids, while in turn Fkbp5 prevents GR activation (40). Besides the observed upregulation of GR expression itself, the lowered *Fkbp5* levels could contribute to a compensatory mechanism by relieving repression of GR function in order to overcome the lack of MR signaling.

Overall, the *Jdp2* gene was the most robust MR target identified in this study. Initially Jdp2 was discovered as a negative regulator of activator protein-1 (AP-1) function, by dimerizing to c-Jun and preventing transcriptional effects (41). Later it was found that Jdp2 can also act in a stimulating fashion, that is as coactivator for the progesterone receptor (42). In this latter study Jdp2 was also shown to have a coactivating effect on transactivation by GR, as was confirmed by Garza et al. (43). We found *Jdp2* to be a bona fide MR target. A feedforward mechanism could be speculated, in which MR can increase Jdp2 levels, which in turn could enhance GR activity. A recent ChIP-seq study in mouse neuroblastoma cells found the Jdp2 binding motif near both MR- and GR-bound sites (44). Besides the differential affinity of MR and GR for their hormone, temporal responses to glucocorticoids could be accounted for by such a feedforward loop. Feedforward models have been described before for GR (45) and other nuclear receptors (46, 47). It is worth noting that Jdp2 has been implicated in AP-1 modulation during fear extinction (48), and polymorphisms in the *Nos1ap* gene have been linked to posttraumatic stress disorder and depression (49), demonstrating also a functional role of these genes in the stress system.

In conclusion, we found three novel hippocampal MR-specific target genes, that are *Jdp2*, *Nos1ap* and *Supv3l1*, of which *Jdp2* is also responsive in an acute stress situation. Dissecting the glucocorticoid response in MR-specific, common and GR-specific pathways will enable us to better understand the stress physiology and pathophysiology of stress-related disorders.

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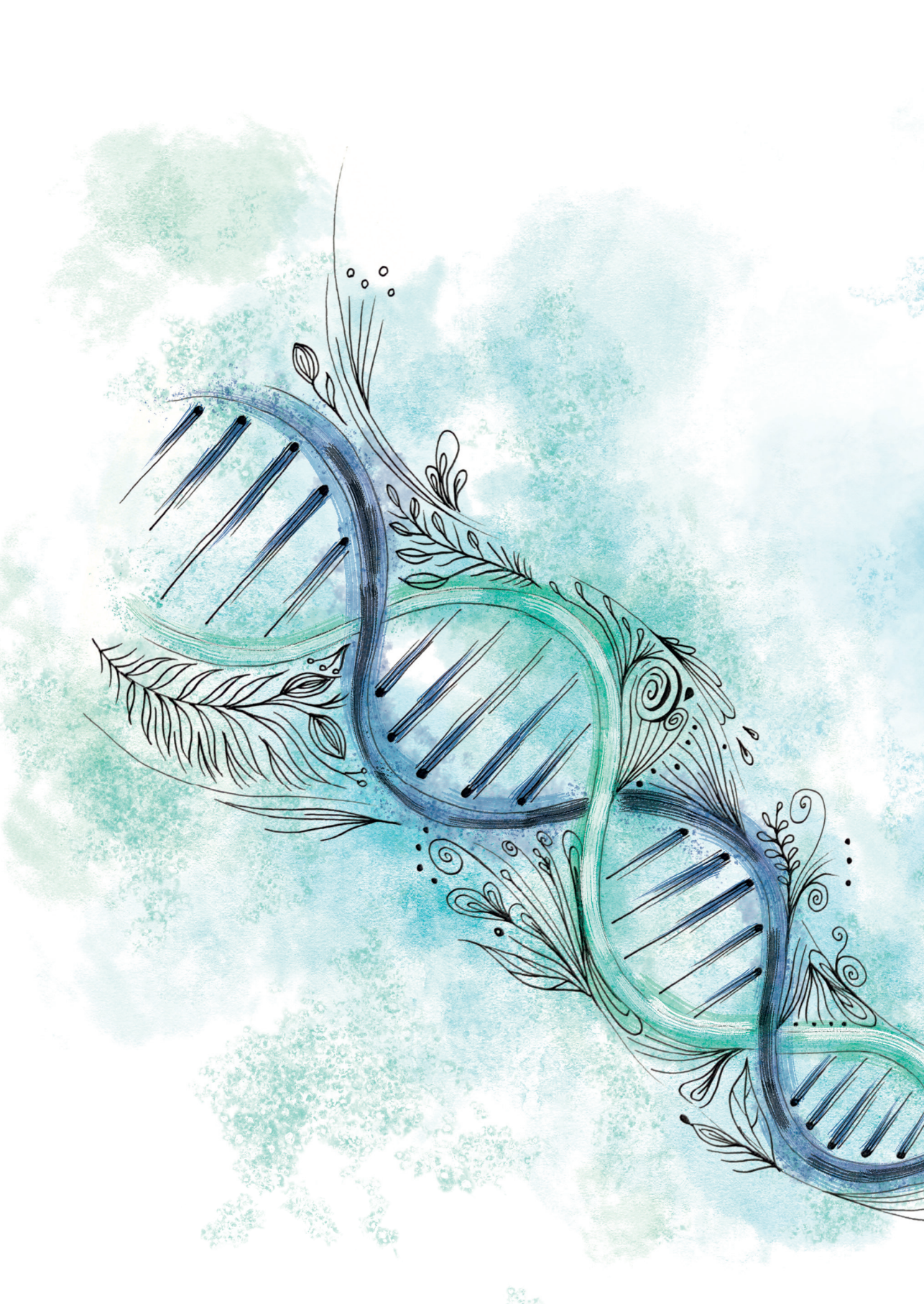

CHAPTER 4

Mechanistic insights in NeuroD potentiation of mineralocorticoid receptor signaling

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Abstract

Mineralocorticoid receptor (MR)-mediated signaling in the brain has been suggested as a protective factor in the development of psychopathology, in particular mood disorders. We recently identified genomic loci at which either MR or the closely related glucocorticoid receptor (GR) binds selectively, and found members of the NeuroD transcription factor family to be specifically associated with MR-bound DNA in the rat hippocampus. We show here using forebrain-specific MR knockout mice that GR binding to MR/GR joint target loci is not affected in any major way in absence of MR. Neurod2 binding was also independent of MR binding. Moreover, functional comparison with MyoD family members indicates that it is the chromatin remodeling aspect of NeuroD, rather than its direct stimulation of transcription that is responsible for potentiation of MR-mediated transcription. These findings suggest that NeuroD acts in a permissive way to enhance MR-mediated transcription, and they argue against competition for DNA binding as a mechanism of MR- over GR-specific binding.

Introduction

The mineralocorticoid receptor (MR) regulates stress coping and has gained significant attention in the field of psychopathology. In general higher brain MR expression levels or MR activity parallel improved cognition and reduced anxiety (1). An MR gain-of-function variant is associated with optimism and provides a decreased risk for depression in females (2). One single nucleotide polymorphism (SNP) that is part of this haplotype affected the cortisol awakening response only in those subjects using antidepressants (3). Furthermore, administration of an MR agonist as a supplement to antidepressant therapy led to faster treatment response (4), and MR activation alone could improve cognitive function in young depressed patients (5). In contrast, chronic stimulation of the highly related glucocorticoid receptor (GR) predisposes to stress-related disorders (6), and GR antagonism seems of benefit in psychotic depression (7). A study combining standard dexamethasone (GR activation) for leukemia treatment with add-on cortisol (concurrent MR activation), shows that MR activity is important for neuronal processes such as sleep cycle and mood regulation (8). It is therefore of great relevance to characterize and enable selective modulation of MR-mediated effects, serving a potential antidepressant approach.

Being part of the nuclear receptor family, MR and GR function as ligand-activated transcription factors, binding the Glucocorticoid Response Element (GRE) at the DNA to mediate transcriptional changes. Even though the two receptors share their ligand cortisol/corticosterone (albeit with a different affinity) and recognize the same motif, receptor-specific binding loci exist as demonstrated in the rat hippocampus (9). This suggests that other factors might be necessary to guide MR/GR-specific binding and subsequent transcriptional effects. We indeed found that binding sites for NeuroD factors were present selectively near MR-bound loci, and confirmed Neurod2 binding near MR-bound but not GR-bound GREs (9). Furthermore NeuroD factors were able to potentiate glucocorticoid-mediated signaling in an *in vitro* setting, although MR/GR specificity was not recapitulated in reporter assays (9).

NeuroD proteins belong to the basic-helix-loop-helix (bHLH) family of transcription factors, and regulate neuronal differentiation. Related MyoD factors are expressed in the muscle, where they induce myogenesis. The bHLH transcription factors bind to E-boxes, which have the sequence CANNTG (10). Specificity is obtained via the middle two nucleotides, with CAGATG known to be a NeuroD-specific binding site, whereas CAGCTG is a shared site that is bound by both MyoD and NeuroD (11). The previously found

interaction between NeuroD and glucocorticoid signaling was based on the presence of the NeuroD-specific motif (9). As the MyoD proteins are better understood in terms of functional domains (12), we also examined transcriptional modulation by bHLH factors at the MyoD/NeuroD shared motif to unravel the interaction between NeuroD and MR here.

The current study aimed to provide mechanistic insights in the NeuroD potentiation of MR signaling, and how MR over GR specificity is achieved. We selected the protein Neurod2 as a representative of the NeuroD family (9). We first questioned whether GR binding would be affected by MR absence, and if Neurod2 binding would be dependent on MR presence. Therefore we assessed GR and Neurod2 binding at previously identified MR targets (9) in the hippocampus of forebrain-specific MR knockout mice (fbMRKO). Subsequently using various E-box binders in a reporter assay, we further explored the mechanism by which NeuroD can enhance glucocorticoid signaling. Our data show that at MR target loci both GR and Neurod2 binding seem independent of MR binding, and it is likely the chromatin remodeling effect of NeuroD that is responsible for the transcriptional potentiation.

Materials and Methods

Animals

Male homozygous forebrain-specific MR knockout ($MR^{flox/flox\_Cre}$, fbMRKO, n=9) and littermate flox heterozygous control mice (MR^{flox/wt_wt}, n=10) (13) aged 10-19 weeks, were housed on a 12-hour light/12-hour dark reversed cycle (lights off at 8:00AM). Mice were group-housed with fbMRKOs and controls combined, and a total of four mice per cage. Each mouse was individually transferred to a novel cage 45 min before harvesting the tissue, in order to ensure GR binding for ChIP analysis. Mice were sacrificed by cervical dislocation around the time of their endogenous corticosterone peak, between 9:00AM-11:30AM. Genotypes were equally distributed over the sacrifice window to prevent an effect by time of the day. Trunk blood was collected, and hippocampal hemispheres were freshly dissected, snap-frozen in liquid nitrogen and stored at -80°C for later analysis. The experiment was performed according to the European Commission Council Directive 2010/63/EU and the Dutch law on animal experiments and approved by the animal ethical committee from Utrecht University.

Plasma corticosterone

Trunk blood was centrifuged for 10 min at 7000*xg*, after which plasma was transferred to new tubes and stored at -20°C for later analysis. Corticosterone levels were determined using an Enzyme ImmunoAssay, according to the manufacturer's instruction (Immunodiagnostic Systems).

ChIP-qPCR

To assess GR and Neurod2 binding at MR-bound loci, we performed chromatin immunoprecipitation (ChIP)-qPCR on hippocampal tissue as described previously (9). Briefly, two fixated hippocampal hemispheres of the same animal were pooled and used for a single ChIP sample (500 µL) to measure GR binding (n=4-5) with 6 µg of anti-GR antibody H-300 (sc-8992X, Santa Cruz) or Neurod2 binding (n=4) with 6 µg of anti-Neurod2 antibody (ab109406, Abcam). Hippocampi were allocated for either GR or Neurod2 detection, with tissue from each group of co-housed mice divided over the two transcription factors. A ChIP using 6 µg of control IgG antibody (ab37415, Abcam) was taken along for background measurements, on a mixed hippocampal chromatin sample per genotype and transcription factor. This was followed by qPCR on undiluted Chelex-isolated (200 µL) ChIP samples, using the primers listed in **Table 1**.

Table 1. Primer sequences used for qPCR on mouse hippocampal ChIP samples. Primers target a mineralocorticoid receptor binding site near the listed gene.

Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
Fkbp5	FK506 binding protein 5	TGCCAGCCACATTCAGAACA TCAAGTGAGTCTGGTCACTGC	122
Kif1c	Kinesin family member 1C	GCTGGGGTGTACACAGATGG TGACTAGCCAGAGCAGTATGTC	156
Klf9	Kruppel-like factor 9	ATCTAGGGCAGTTTGTTCAA GGCAGGTTCATCTGAGGACA	96
Per1	Period circadian clock 1	GGAGGCGCCAAGGCTGAGTG CGGCCAGCGCACTAGGGAAC	73
Rilpl1	Rab interacting lysosomal protein-like 1	CAGGCAGATGCCAGGCT CCCATGCCTGTTCCTCTAGT	106
Zfp219	Zinc finger protein 219	AGTCCATCACATTCTGTTGCTTTC TAGTCAGCTATGACCATGCAGT	131

Reporter assays

For mechanistic insights into the role of NeuroD factors on MR/GR-driven promoter activity, we performed luciferase reporter as described previously (9). In short, HEK293 cells were transfected using FuGENE (Promega) with luciferase construct (GRE-At, 30 ng/well), expression vector for either MR or GR (10 ng/well), with or without NeuroD/MyoD cofactor (10 ng/well), and Renilla (1 ng/well) for normalization. To exclude glucocorticoid effects from the medium we used charcoal-stripped fetal bovine serum (Sigma) during the experiments. After 24 hours stimulation of the cells with 10^{-7} M corticosterone (Sigma) reporter protein levels were measured using the Dual Luciferase Reporter Assay System according to the manufacturer's instruction (Promega).

Plasmids

Transcriptional activity was assessed at a GRE-driven promoter combined with either the NeuroD-specific (CAGATG) or the MyoD/NeuroD-shared (CAGCTG) motif. The GRE and NeuroD binding site-containing vector (GRE-At_GA) was constructed before (GRE-At-pGL4 (9)). For the generation of the GRE-At_GC luciferase construct, we exploited mutagenesis targeting the NeuroD binding site (GA>GC) using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). PAGE-purified mutagenic primers were: 5'-CTCGAGGATGGCAGCTGGAGCTAAGAACAGAA-3' and 5'-TTCTGTTCTTAGCTCCAGCTGCCATCCTCGAG-3'. For MR and GR expression we used the 6RMR and 6RGR-based plasmids (14). Expression vectors (all pCS2) for Neurod2, MyoD, a chimera of MyoD with the DNA-binding domain of Neurod2 (MyoD(ND2bHLH)), MyoD lacking the N-terminal domain (MyoDΔN) and Myf5 were kindly provided by Dr. Tapscott (12, 15).

Statistics

On the ChIP data we ran unpaired *t*-tests with Holm-Sidak multiple comparison correction. For the reporter assays we performed statistics on the fold induction by ligand (calculated for each corticosterone-treated sample as signal in the presence of hormone divided by the average signal from the same condition in absence of hormone). The first reporter experiment (different cofactors at various concentrations) was analyzed by two-way ANOVA; the second reporter experiment (different cofactors) was analyzed by one-way ANOVA, both followed by Tukey's post-hoc tests. All data are presented as mean ± standard error of the mean.

Results

DNA binding assessed by ChIP-qPCR

In order to define the mechanism behind the NeuroD potentiation of glucocorticoid signaling in more detail, we first tested whether MR binding to its hippocampal DNA targets affects local GR and Neurod2 binding. Although family members Neurod1, Neurod2 and Neurod6 are all expressed in the adult mouse hippocampus and are able to bind the same NeuroD binding site (9), we focus here on Neurod2. GR and Neurod2 occupancy of MR-binding loci was measured by ChIP-qPCR on hippocampus of WT and fbMRKO mice. The fbMRKO mice show ablated hippocampal MR mRNA levels (16), which is accompanied by efficient knockdown of MR protein (17). Plasma corticosterone of all animals was over 140 ng/mL, ensuring ligand occupancy of both MR and GR (18). No difference in corticosterone plasma levels was observed between the two genotypes, with an average of 363 ± 30 ng/mL for WT mice and 313 ± 44 ng/mL for fbMRKO mice (**Figure 1A**).

MR effect on GR binding

We aimed to investigate if the joint binding of MR and NeuroD on the DNA is related to competition for GR binding at the same locus. GR binding was confirmed in WT mice for classical glucocorticoid target genes *Fkbp5* and *Per1* (**Figure 1B**), which are occupied by both MR and GR (19). Other MR-GR overlapping loci near the *Klf9* (20) and *Kif1c* (9) genes showed evident GR binding. Previously identified MR-specific target *Rilpl1* (9) showed low GR signal, to the same extent as MR-GR overlapping target *Zfp219* (9). GR binding levels were similar in the fbMRKO mice for most of the genes measured, suggesting that GR binding is not dependent on MR binding at these target loci. Only the GR binding at *Per1* was slightly enhanced in MR absence ($P = 0.00055$), which might point to a compensatory mechanism at this specific binding site. However, in general GR binding does not seem to compensate for the lack of MR binding in fbMRKO mice.

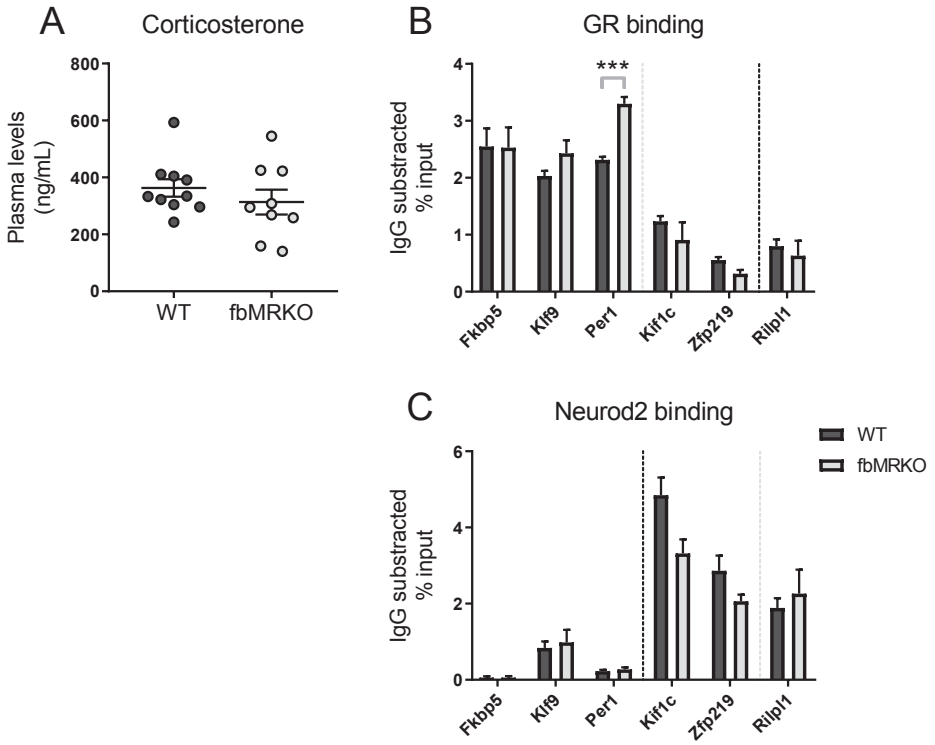

Figure 1. **A)** Corticosterone levels of wild-type (WT) and forebrain-specific mineralocorticoid receptor (MR) knockout (fbMRKO) mice. In these mice chromatin immunoprecipitation coupled with quantitative polymerase chain reaction (ChIP-qPCR) measurements for **B)** glucocorticoid receptor (GR) and **C)** Neurod2 were performed. For each gene, the corresponding immunoglobulin G (IgG) background signal is subtracted from detected binding levels, expressed as the percentage of immunoprecipitated DNA. The binding sites near *Fkbp5*, *Klf9*, *Per1*, *Kif1c* and *Zfp219* are joint MR/GR loci, while *Rilpl1* has been identified as an MR-specific target (9) (separated by the right dotted line). Genes are further sorted based on the absence (*Fkbp5*, *Klf9*, *Per1*) or presence (*Kif1c*, *Zfp219*, *Rilpl1*) of a NeuroD binding sequence near the MR binding site (separated by the left dotted line). *** $P<0.001$

MR effect on Neurod2 binding

Next, we addressed the question whether the association between MR and NeuroD factors that we observed previously implies that Neurod2 binding at these loci depends on the presence of MR. We measured Neurod2 binding at the same loci as for GR binding. No Neurod2 binding motif was detected in the ChIP-identified MR-GR overlapping binding sequences near *Fkbp5*, *Klf9* and *Per1*. For the *Kif1c* and *Zfp219* associated MR-GR overlapping loci a directed motif search (9) did reveal a Neurod2 binding motif. Neurod2 binding was indeed observed for *Kif1c*, *Zfp219* and to a lesser extent in *Klf9*, and for MR-specific *Rilpl1* as observed before (9) (**Figure 1C**). Those genes with relatively

low GR binding showed higher Neurod2 binding and vice versa, supporting the earlier finding that Neurod2 seems to interact preferentially with MR (9). The fbMRKO mice demonstrated unchanged Neurod2 binding levels, indicating the presence of MR is not crucial for Neurod2 binding. For *Kif1c* there might be an interaction, as the Neurod2 signal seems to be lower in fbMRKO compared to WT animals, but this difference does not statistically hold after multiple comparison correction ($P = 0.23$). Overall, these data show that Neurod2 binding to MR-associated loci is independent of MR binding.

Structure-function relationship

We continued unraveling the mechanism behind the NeuroD potentiation of glucocorticoid signaling by exploring which coactivation property of the NeuroD protein is responsible for the transcriptional potentiating effects. While the structure-function relationship of the NeuroD family is not known in detail, much more is known about the related bHLH family of MyoD proteins (12). We therefore used the myogenic regulatory factors MyoD and Myf5 as tools to study the effect of bHLH factors in the potentiation of glucocorticoid signaling. Where MyoD can induce both histone acetylation at H4 (chromatin remodeling) and in addition recruit RNA polymerase II (direct activation mediated by the transcriptional activation domain), Myf5 is only able to induce H4 acetylation as a manner to enhance transcription (12). NeuroD family members have been shown to affect both chromatin accessibility and direct transcriptional activation (11, 21), although these functions have not been assigned to a specific part of the protein. Comparing the myogenic variants will enable us to dissect the process important for the potentiation of glucocorticoid signaling.

Transcriptional potentiation by MyoD

We started by exploring whether MyoD is able to show a similar coactivation effect for MR/GR-mediated signaling as Neurod2 did in our reporter assay. Despite the *in vivo* binding selectivity of Neurod2 with MR (and not GR), Neurod2 exhibits coactivation of MR but also GR transcriptional activity *in vitro* (9). MyoD and NeuroD have both unique and common response elements (11). Our original reporter construct that is based on *in vivo* MR ChIP-sequencing binding sites (9), harbors the NeuroD-specific CAGATG along a GRE. In a first experiment we tested the effect of Neurod2, MyoD and a chimeric MyoD protein with its bHLH domain substituted by that of Neurod2 (MyoD(ND2bHLH)) in the concentrations of 1-3-10 ng/well (**Figure 2**). Both a cofactor ($F_{2,24} = 356.3$ for MR; $F_{2,24} = 708.3$ for GR, both $P < 0.000001$) and concentration ($F_{3,24} = 247.6$ for MR; $F_{3,24} = 489.0$ for

GR, both $P < 0.000001$) effect, plus an interaction ($F_{6,24} = 71.0$ for MR; $F_{6,24} = 159.2$ for GR, both $P < 0.000001$) were observed.

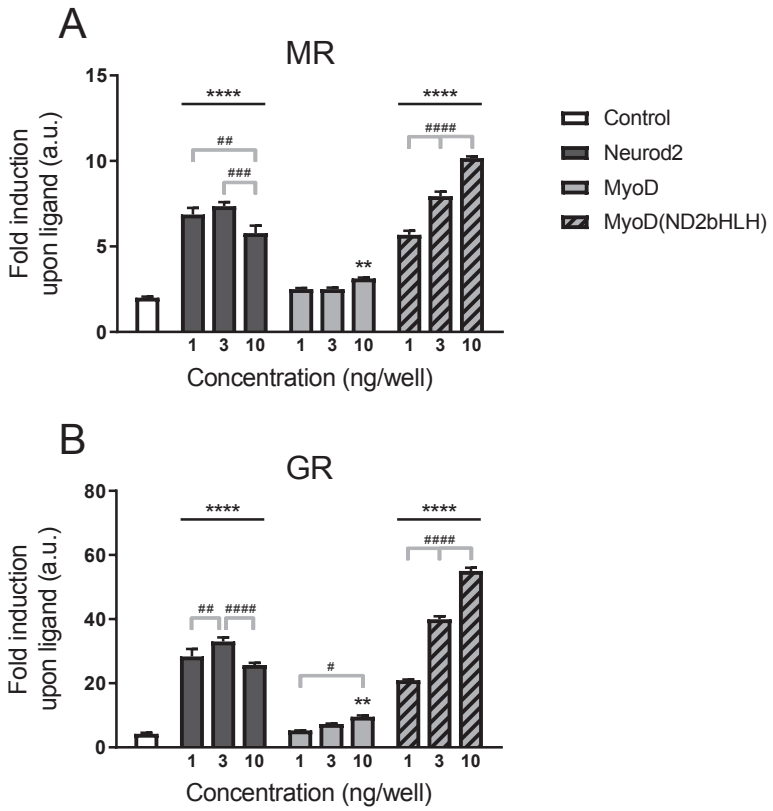

Figure 2. Specificity of NeuroD coactivation at the previously identified binding motif (CAGATG) for **A)** MR and **B)** GR. HEK293 cells were transfected with GRE-At_GA luciferase construct, MR or GR (10 ng/well), various amounts of Neurod2, MyoD or the MyoD/Neurod2 chimera (MyoD(ND2bHLH)) (1-3-10 ng/well), and stimulated with corticosterone (10^{-7} M). Data are presented as luciferase activity fold induction upon corticosterone treatment. a.u. = arbitrary unit; ** P<0.01, **** P<0.0001 compared to control condition; # P<0.05, ## P<0.01, ### P<0.001, #### P<0.0001 for within group comparisons

We confirmed Neurod2 could potentiate glucocorticoid signaling for both MR and GR (**Figure 2A, 2B**). The observed Neurod2 effect was receptor-mediated, as in absence or with lower amounts of nuclear receptor expression vector Neurod2 did not enhance the glucocorticoid-dependent transcriptional increase (**Supplemental Figure 1**). We showed that also MyoD can potentiate MR- and GR-mediated transcriptional activity, once brought to the DNA. Coactivation by MyoD itself is minimal with a slightly higher

fold induction in the upper tested dose compared to control cells without cofactor (*P* = 0.0062 for MR; *P* = 0.0019 for GR), but can be enhanced to an extent similar to Neurod2 by swopping the MyoD DNA-binding domain (DBD) with that of Neurod2 as demonstrated using the MyoD(ND2bHLH) chimera (**Figure 2**). In its highest tested dose the chimera could even potentiate glucocorticoid signaling to a superior extent. Of note, the chimera showed a clear dose-dependent increase in potentiation over the concentration range tested. These findings indicate the Neurod2 DBD is required for coactivation, and the DNA sequence rather than the bHLH protein function drives specificity.

Activation domain not crucial for potentiation

Finally we tested several bHLH factors for their coactivation ability in our reporter assay to examine the contribution of different protein domains. In order to have a fair comparison of all variants, we ensured a similar binding affinity of NeuroD and MyoD by further studying a reporter construct containing the shared CAGCTG motif (11). At this reporter Neurod2 and MyoD could potentiate MR signaling to the same extent (**Figure 3A**), while for GR-mediated transcription the MyoD potentiation was somewhat lower than by Neurod2 (*P* = 0.000003, **Figure 3B**). MyoD lacking its activation domain (MyoDΔN) demonstrated a less strong potentiation of GR-mediated signaling compared to full length MyoD (*P* = 0.0012), as did family member Myf5 (*P* = 0.0035), but both MyoDΔN (*P* = 0.047) and Myf5 (*P* = 0.016) still showed a significantly higher transcriptional effect upon corticosterone treatment than the control condition without overexpression (**Figure 3B**).

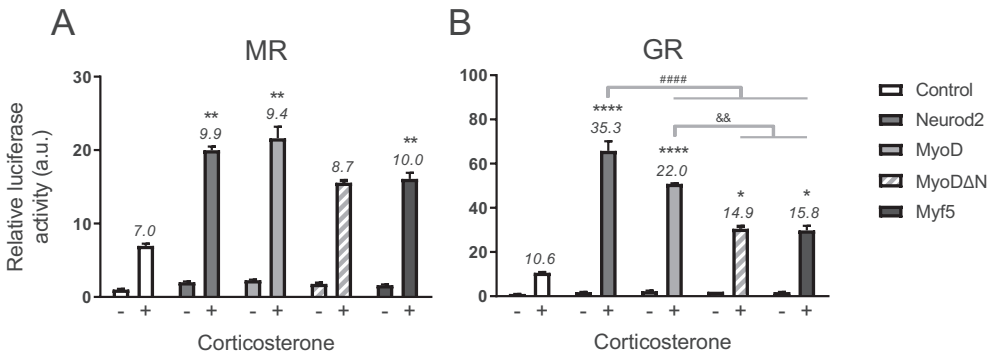

Figure 3. Modulation by NeuroD and MyoD variants at the shared binding motif (CAGCTG) for **A)** MR- and **B)** GR-mediated transcription. HEK293 cells were transfected with GRE-At_GC luciferase construct, MR or GR (10 ng/well), and Neurod2, MyoD, MyoDΔN or Myf5 (10 ng/well), and stimulated with corticosterone (10^{-7} M). Luciferase activity of nonstimulated control cells was normalized to 1. Numbers represent fold induction upon corticosterone treatment. a.u. = arbitrary unit; * *P*<0.05, ** *P*<0.01, **** *P*<0.0001 compared to control condition; #### *P*<0.0001 compared to Neurod2 condition; && *P*<0.01 compared to MyoD condition

The effect of the bHLH proteins on MR transactivation was more modest. Interestingly, the MyoDΔN and Myf5 coactivating potential for MR-mediated signaling was not different from Neurod2 and MyoD (**Figure 3A**). However, MyoDΔN did not reach significance in corticosterone induction compared to control cells (**Figure 3A**). Although potentiation of GR transcriptional activity by bHLH factors seems thus partly dependent on their activation domain, these data suggest that the coactivation of MR signaling by Neurod2 postulated to happen *in vivo* (9) is likely mediated via chromatin remodeling rather than direct transcriptional activation.

Discussion

This study further elucidates the mechanism behind NeuroD potentiation of brain MR signaling. First transcription factor DNA binding was assessed by ChIP-qPCR in mice lacking MR in (amongst other brain regions) their hippocampus. Both GR and Neurod2 binding were not altered in these fbMRKO mice compared to control mice, except for an enhanced GR signal at the *Per1* promoter in absence of MR. Subsequently bHLH factors of the NeuroD and MyoD families were used to study coactivator effects in an MR/GR-driven reporter assay. Those factors lacking (MyoDΔN) or with diminished (Myf5) activator function were able to potentiate the glucocorticoid-stimulated transcriptional activation as well as Neurod2 and MyoD in case of MR-dependent transcription, suggesting coactivation of MR signaling by Neurod2 does not require its activation domain.

Effects on DNA binding

Because MR and GR can bind the same DNA sequences, GREs, absence of MR might affect genomic binding by GR. Competition between MR and GR at a specific locus does not seem to play a major role, as there was no overall enhanced GR binding in the fbMRKO mice at the sites we examined, even though hippocampal GR expression is upregulated in these animals (13, 16). Only in the case of *Per1*, higher GR occupancy levels were observed at the promoter region in absence of MR. At this locus it has been demonstrated that besides homodimerization, MR and GR can combine to form heterodimers (19). We can however not distinguish between these two binding modes in our measurements. The increased GR binding could reflect a compensatory mechanism to maintain a required degree of *Per1* expression and is in agreement with the fact that basal *Per1* mRNA levels were not altered in fbMRKO mice (16). Rather than competition, data on joint occupancy suggest there can be synergism between two transcription factors binding the same site,

via a process called 'assisted loading'. For concurrent stimulation of the GR and estrogen receptor (ER; where ER is altered to also recognize the GRE), GR activation could enhance ER binding at the same locus (22). In the present study GR binding is not significantly diminished when MR is lacking, suggesting such assisted loading is not applicable for MR-GR joint loci here. In our measurements of whole hippocampus we should acknowledge that we work under the assumption that all studied cells have (similar amounts of) MR and GR, but effects on DNA binding could be diluted as MR/GR expression is not homogeneous throughout the hippocampal regions and in the various cell types present (23). Single cell analysis will offer a solution to study transcription biology in a cell-type specific manner (24). Nevertheless, our data indicate that GR binding is predominantly independent from MR presence in the hippocampus.

In the same setting we studied if Neurod2 binding was affected by absence of MR. No differences in Neurod2 signal at the MR target loci were observed in MR deficient mice, which implies that NeuroD facilitates MR binding in a unidirectional manner. We cannot exclude the possibility that Neurod2 binding is affected by or dependent on changes in stress hormone levels, since this was not studied here. Presence of another collaborative transcription factor (nuclear factor-1) found near preaccessible GR-bound loci was independent of corticosterone treatment or exposure to restraint stress (25). As discussed below, our reporter assay data suggest that the potentiation of MR signaling by NeuroD is likely mediated via chromatin accessibility.

Mechanism of glucocorticoid signaling potentiation

Unfortunately the NeuroD activation domain is not well documented/distinguished, but MyoD family members do have well described domains (12). We first tested whether MyoD was able to potentiate glucocorticoid signaling at a reporter construct containing a GRE and NeuroD-specific E-box (CAGATG). When the MyoD DBD was adapted to that of Neurod2 in order to bind this motif efficiently, MyoD could coactivate glucocorticoid-mediated signaling to a similar (or even superior) extent as Neurod2. This is in line with findings by Fong et al., showing that MyoD could be redirected to NeuroD target sites through replacement of its bHLH domain by the analogue sequence of Neurod2 and hereby could activate part of the neuronal differentiation program (15). The same group has demonstrated that NeuroD and MyoD can bind and drive transcription at the E-box that is specific for the other bHLH factor, but have a strong preference for their specific motifs (11, 15). This explains why unmodified MyoD showed a slight transcriptional potentiation on the NeuroD-specific binding site at its highest concentration tested. In

concordance with the DBD being decisive in converting MyoD into a neurogenic factor (15), the specificity of the interaction between NeuroD/MyoD and MR/GR in our data is also determined by the ability of the factor to bind the DNA rather than a protein-specific functionality. Interactions between bHLH transcription factors and steroid receptors can be speculated to be generic but have cell/tissue-type dependent mechanisms. For instance, bHLH proteins DEC1/DEC2 (differentiated embryo chondrocyte) were found to corepress liver retinoid X receptors (26). Likewise, E47 can modulate hepatic glucocorticoid action by promoting GR occupancy of metabolic target loci (27). Of relevance in the testis, Pod-1 (also: transcription factor 21) could diminish transactivation by the androgen receptor (28).

For unbiased comparisons we proceeded our experiments with a reporter construct containing the shared E-box (CAGCTG), which is bound with similar affinity by both Neurod2 and MyoD (15). Coregulators can modulate transcription by affecting chromatin accessibility and/or recruitment and stabilization of the transcriptional machinery (29). To distinguish between these two modes, we made use of a truncated version of MyoD lacking its activation domain (responsible for direct recruitment), and the myogenic Myf5 that has a weak activation domain (and therefore relies mainly on its chromatin remodeling ability) compared to MyoD (12). All MyoD variants were able to coactivate the GRE-driven reporter. Strikingly, while potentiation of GR signaling was partly dependent on the bHLH activation domain, coactivation of MR signaling was almost unaffected when using the factors with diminished direct transcriptional activation. Extrapolating these findings to the NeuroD family, the chromatin remodeling aspect of NeuroD seems thus sufficient for effective potentiation of MR-mediated signaling. This is in accordance with the pioneer function of family member Neurod1 demonstrated in a ChIP-sequencing experiment on developing neurons (21). Of note, during neurogenesis occupancy of the Neurod2-specific motif was linked to gene expression effects, while the shared motif related mostly to chromatin modifications (11). Despite the fact that transient systems might be considered to have an undefined chromatin context, it has been shown that exogenous plasmids do interact with endogenous histone proteins (30, 31) and can serve as a proper model to study effects mediated via chromatin accessibility as observed here.

MR selective signaling and future implications

A number of issues have remained unaddressed. In the current study we have been looking at only a subset of Neurod2 sites, and mainly focused on targets bound by both MR and GR. It would be of interest to study genome-wide effects and observe if MR-

specific sites become GR-bound in absence of MR. We also have to point out that we have not assessed *in vivo* which NeuroD factor(s) is/are responsible for potentiation of MR signaling, as we only measured and detected Neurod2 binding at MR-bound sites (9). The basis for MR over GR specificity in full chromatin is not known, but the fact that bHLH chromatin remodeling plays a more important role in case of MR-mediated reporter activation is in line with the fact that we could correlate MR and Neurod2 binding *in vivo* (9). Besides, those MR target genes with relatively low GR signal had high Neurod2 binding in our current ChIP data. A study by Pooley et al. found that 17% of GR-bound loci contained a NeuroD binding site in their vicinity (25). These are likely MR/GR joint sites comparable to the here studied ones, some of which do show an E-box and could be co-bound by Neurod2. MyoD family inhibitor domain-containing protein (MDFIC) has been found to bind the hinge region of unliganded GR, is capable of regulating GR phosphorylation and can by this means define the receptor transcriptome (32). This interaction might play a role in the MR/GR binding selectivity near Neurod2-bound sites, as our earlier studies suggested that proteins in the nuclear receptor complex might account for the MR preference (9). One promising approach to further elucidate the MR over GR specificity would be to have ChIP experiments followed-up by proteomics (33).

The question emerges what the NeuroD potentiation of MR signaling implicates for stress processing and stress-related disorders. Increased *Neurod2* expression levels were detected in the ventromedial prefrontal cortex of men with major depressive disorder compared to healthy control subjects (34). In a mouse model of chronic social defeat paradigm, overexpression of Neurod2 in the ventral hippocampus reduces, while overexpression in the nucleus accumbens increases social interaction time (35). Antidepressant agomelatine could normalize the raise in hippocampal *Neurod1* expression of mice that underwent chronic mild stress (36). Furthermore, fish in touristic zones were shown to express higher levels of *Neurod1* and the MR gene *Nr3c2* relative to fish at control sites (37). Together these observations strongly suggest a functional and context-dependent link between NeuroD and stress regulation. How this might depend on MR or influence MR function remains to be investigated. Further research is needed focusing on the *in vivo* specificity of the interaction between MR and NeuroD, and directionality in the highly adaptable stress system. MR activation is considered a promising strategy to promote stress resilience (1). It would be of great interest to test if SNPs in the MR gene can affect NeuroD potentiation. In conclusion, we show that GR and Neurod2 binding at MR target loci is not dependent on MR presence and that Neurod2 potentiation of MR signaling is likely mediated via chromatin remodeling. We summarized the findings of

this study in **Figure 4**. Future studies will have to point out how the interaction between Neurod2 and MR might be exploited to modulate MR-specific effects in the brain and affect associated behavior.

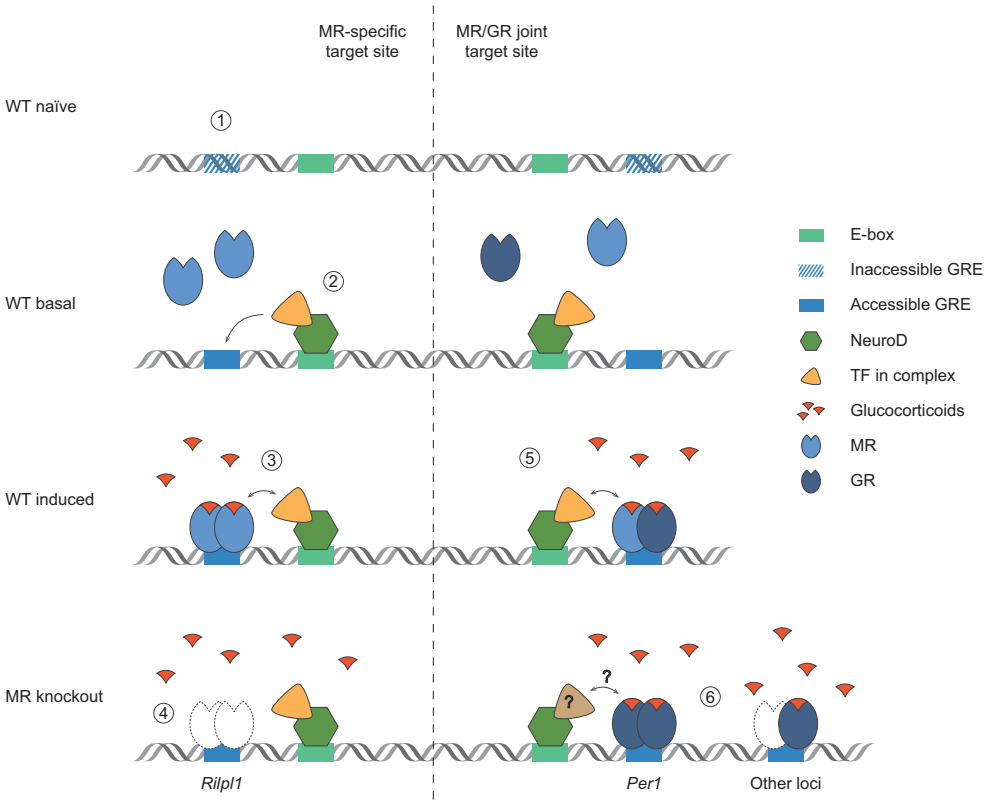

Figure 4. Summary of the interaction between hippocampal MR and NeuroD. GREs previously inaccessible **[1]** could be rendered accessible by chromatin remodeling (one-way arrow) induced by NeuroD **[2]** binding at a nearby E-box (the NeuroD-specific sequence CAGATG). Upon ligand availability MR can bind an accessible GRE **[3]** in order to modulate transcriptional activity of its target genes. This interaction between NeuroD and MR (two-way arrow) is likely mediated via additional TF(s) in the transcriptional complex (9). In forebrain MR knockout mice **[4]** GR is not compensating for the lack of MR binding at the MR-specific *Rilpl1* site. Also at several MR/GR joint target sites **[5]** NeuroD occupancy is observed in the vicinity. Of note, we cannot discriminate between the binding of homo- and heterodimers in the present study. In absence of MR **[6]** GR binding is increased at the *Per1* promoter, while for the other tested loci GR binding levels are unaltered. For sites that become GR-specific due to MR knockout, interactions with NeuroD remain to be explored, and other TF(s) might be involved **[?]**. MR = mineralocorticoid receptor, GR = glucocorticoid receptor, GRE = glucocorticoid response element, TF = transcription factor, WT = wild type

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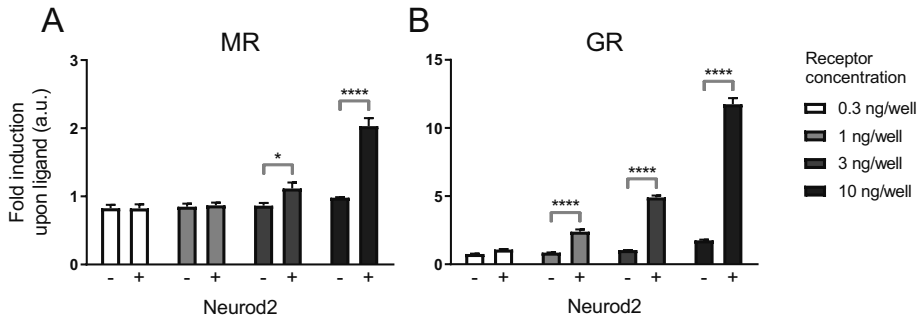
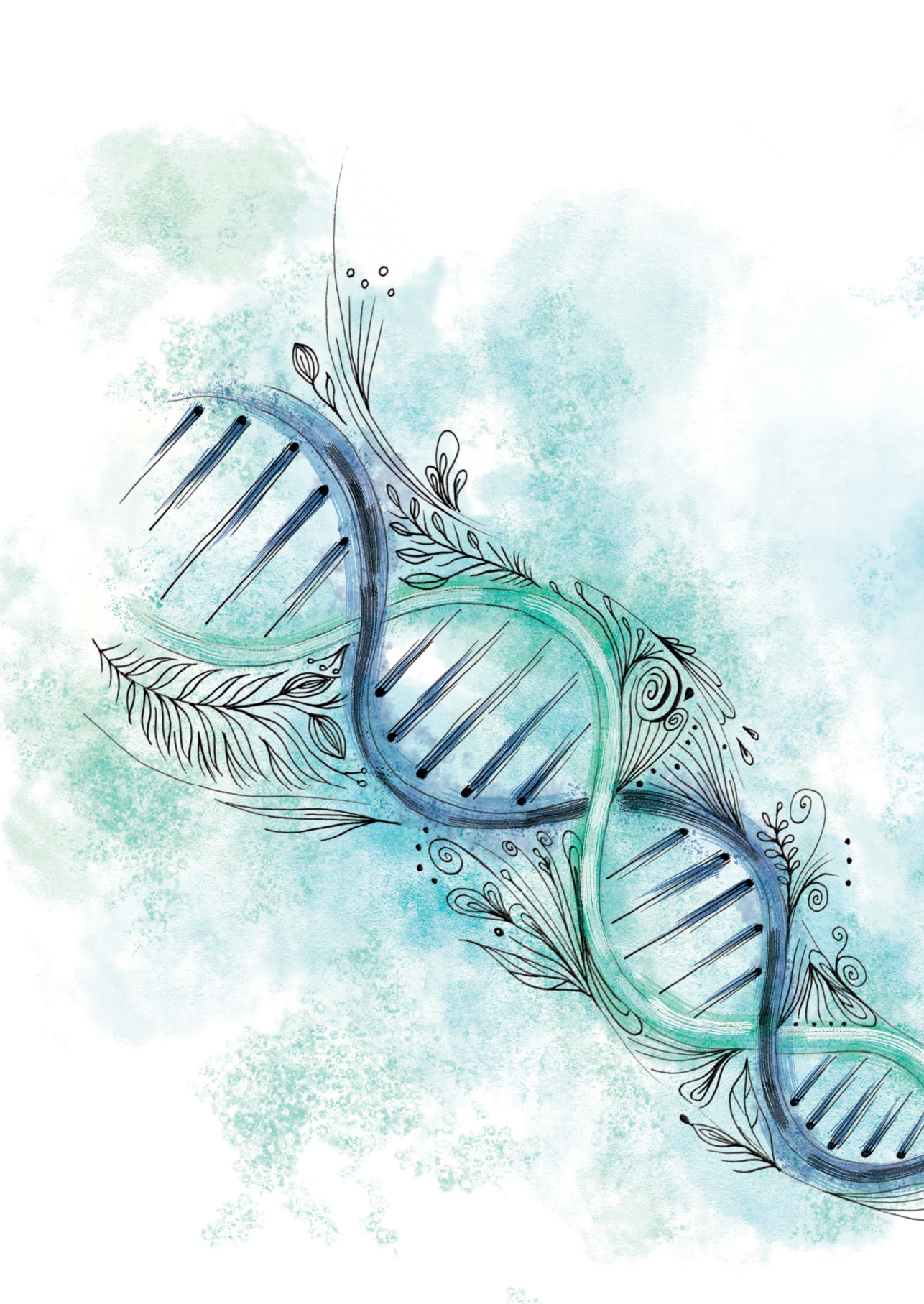
Supplemental data



Supplemental Figure 1. Neurod2 effect as a result of nuclear receptor titration for **(A)** MR and **(B)** GR. HEK293 cells were transfected with GRE-At_GC luciferase construct, and various amounts of MR or GR (0.3-1-3-10 ng/well), with or without Neurod2 (10 ng/well), and stimulated with corticosterone (10^{-7} M). Data are presented as luciferase activity fold induction upon corticosterone treatment. a.u. = arbitrary unit; * P<0.05, **** P<0.0001



CHAPTER 5

Interactions of transcription factors at the genome induced by stress hormones during memory consolidation

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Abstract

Emotionally charged events are remembered better than neutral ones, but the exact mechanism by which this comes about is unknown. Potentiation of memory formation by emotions depends on the synergistic action of stress hormones (nor)adrenaline and glucocorticoids on defined circuits in the brain. The associated intracellular pathways converge on two transcription factors: phosphorylated cAMP response element-binding protein (pCREB) and the glucocorticoid receptor (GR), respectively. Our hypothesis is that there are interactions between pCREB and GR at the genome during the consolidation of arousing learning conditions, and as a consequence the GR cistrome is affected by introduction of a memory task context.

To model emotional learning, we used an object location memory (OLM) task in rats combined with systemic corticosterone (CORT) administration. CORT injected immediately after training led to a dose-dependent enhancement of memory formation, with rats administered the higher dose of 3.0 mg/kg exhibiting better 24-hour retention than non-discriminating vehicle-injected rats. To map whole-genome pCREB and GR binding during the memory consolidation process, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) on hippocampal tissue of rats sacrificed 45 minutes after OLM training and/or injection of either vehicle or 3.0 mg/kg CORT.

In the current analysis we focused on the GR data: 58 genomic loci showed higher GR binding upon CORT injection, and for 8 loci we found reduced GR occupancy. OLM training partially affected the subset of differentially bound GR sites. We confirmed CORT-induced activation of classical GR target *FK506 binding protein 5* (*Fkbp5*) independent of the training status of the rats. In addition *Gap junction protein, beta 6* (*Gjb6*) and *NMDA receptor synaptonuclear signaling and neuronal migration factor* (*Nsmf*) were identified as novel GR targets. The data support the existence of both training context-dependent and -independent GR binding in the hippocampus after CORT treatment in adrenally intact rats.

Introduction

Enhanced memory formation of emotional events is brought about with help of two different stress hormones (1, 2). On the one hand, there is the acutely acting adrenaline that translates into release of noradrenaline in the brain. On the other hand, glucocorticoids (cortisol in humans, corticosterone (CORT) in rodents) play a role with some delay in their rise and action upon a stressful event. In the object recognition memory task (ORM) in rodents, CORT can serve as a switch for the encoding of an otherwise more neutral event. Administration of CORT directly after training enhances the consolidation process in a setup where vehicle treated animals do not show long-term memory (3). This memory enhancement is dependent on the glucocorticoid receptor (GR), presumably in part via its transcriptional effects, based on evidence from similar effects in a different spatial learning task (4, 5). However, any underlying mechanism at the level of the genome remains to be elucidated.

CORT-induced potentiation of memory in (amongst others) the ORM task requires noradrenaline signaling in the brain (6-9). This is in agreement with the fact that glucocorticoid transcriptional effects depend on (cellular) context (10, 11), and suggests that the signaling pathways linked to noradrenaline and CORT interact at some point during the memory formation process (12). In order to examine genomic interactions between the two signaling pathways, the current study made use of the object location memory (OLM) task. In this task the location of one of the objects (compared to the type of object in ORM) is changed between training and testing phase. Memory in the OLM task is dependent on the hippocampus, the brain region responsible for processing spatial information (13, 14). Whereas novelty-induced noradrenaline signaling is stimulated endogenously upon the low-arousing first encounter of the training apparatus (3, 8), glucocorticoids are injected directly after training to mimic an emotional event and induce concomitant memory (i.e. preference for the newly located object over the familiarly positioned object).

Noradrenaline stimulates, amongst other pathways, the transcription factor cAMP response element-binding protein (CREB) by phosphorylation, leading to phospho-CREB (pCREB) (15). CORT activates the GR, a nuclear receptor that modulates the transcription of its target genes (16). Although a membrane variant of the GR has been shown to be of relevance in the formation of long-term memory (13), we focus here on the DNA binding receptor. Both pCREB and GR are important for (spatial) memory formation (5, 17-20) and as transcription factors (TFs) bind to specific DNA sequence motifs. We hypothesized

that pCREB and GR (as downstream targets of noradrenaline and CORT, respectively) interact on the DNA level during memory consolidation, either by direct protein-protein interactions (21), chromatin remodeling (pioneering) (18, 22) or complex stabilization (23). As a result of such interactions, we expect the GR binding intensity and/or set of GR-bound loci to be learning context dependent, i.e. different between trained animals compared to non-trained controls.

We determined genome-wide binding of pCREB and GR in the hippocampus by chromatin immunoprecipitation followed by sequencing (ChIP-seq), in an OLM setup in which CREB and GR were stimulated separately or combined. Downstream analysis focused on GR data, which confirmed the classical glucocorticoid target gene *FK506 binding protein 5* (*Fkbp5*) and disclosed the novel target genes *Gap junction protein, beta 6* (*Gjb6*) and *NMDA receptor synaptonuclear signaling and neuronal migration factor* (*Nsmf*). Partial overlap of CORT-mediated changes on the GR cistrome found in trained versus non-trained animals, points towards context-dependent GR binding for at least a limited subset of target genes. Follow-up research might reveal additional target genes in the context of the OLM task and will need to elaborate on the role of these genes specifically during emotional enhancement of memory consolidation.

Material and methods

Animals

Male Sprague-Dawley rats (340-400 g at time of training) from Charles River Laboratories (Germany) were individually housed in a temperature-controlled (22°C) vivarium room at a 12-h light/12-h dark cycle (lights on at 7:00AM). Food and water were available *ad libitum*. Training and testing were performed during the light phase of the cycle between 10:00AM-3:00PM. All procedures were in compliance with the European Communities Council Directive on the use of laboratory animals (2010/63/EU) and the Dutch law on animal experiments and were approved by the Animal Ethics Committee of Radboud University, Nijmegen, The Netherlands.

Object location memory task

The experimental apparatus for the OLM task was a grey open-field box (40 x 40 x 40 cm) with a sawdust-covered floor, placed in a dimly illuminated room. The objects to be explored were white glass light bulbs (6 cm diameter by 11 cm length) and transparent

glass vials (5.5 cm diameter by 5 cm height). Five consecutive days before training rats were handled and a subcutaneous injection was mimicked to habituate the animals to drug administration. To ensure training-induced arousal and endogenous noradrenaline activation, the rats were not habituated to the OLM box prior to the training session (3, 8). On the training trial, the rat was placed in the experimental apparatus and allowed to explore two identical, symmetrically placed objects (A1 and A2) for 3 minutes. To avoid the presence of olfactory trails, sawdust was stirred and the objects were thoroughly cleaned with 70% ethanol between rats. CORT (0.3, 1.0 or 3.0 mg/kg; Sigma-Aldrich) dissolved in 5% ethanol in saline, or vehicle, was administered subcutaneously (2.0 mL/kg) immediately after training, and the rat was returned to its home cage.

Retention was tested 24 hours later. Two copies of the familiar object (A3 and A4) were placed in the box, of which one in the same location as during training and the other in a novel location. All combinations and locations of objects were counterbalanced to reduce potential bias because of preference for particular locations or objects. The rat was placed in the experimental apparatus for 3 minutes and behavior was recorded by a camera mounted above the box, for later offline analysis. Rat behavior was analyzed with The Observer XT software (Noldus Information Technology). The time spent exploring each object was measured. Furthermore rearing, freezing and the amount of quadrant crossings (as a measure of activity) were scored. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. Turning around, nibbling, climbing or sitting on an object was not considered exploration. A discrimination index was calculated as the difference in time exploring the object in the novel and familiar location, expressed as the ratio of the total time spent exploring both objects. Rats showing a total exploration time <8 s on either training or testing were excluded from further analysis.

Two batches of animals were trained and tested in the OLM task to establish the optimal memory-enhancing CORT dose. For the ChIP-sequencing and validation experiment, rats were either trained on the OLM task or not, followed by a subcutaneous vehicle or CORT (3.0 mg/kg) injection, and sacrificed 45 minutes afterwards. This resulted in four experimental groups: non-trained vehicle-injected, non-trained CORT-injected, OLM-trained vehicle injected and OLM-trained CORT-injected. From these rats, trunk blood was collected with 300 µL 0.1 M EDTA for assessment of plasma CORT levels. Hippocampal hemispheres were freshly dissected, cut into smaller pieces (for ChIP only), snap-frozen in liquid nitrogen and stored at -80°C until later processing.

Plasma corticosterone

Trunk blood was centrifuged at 3000x*g* for 15 minutes, after which plasma was transferred to new tubes and stored at -20°C for later analysis. CORT levels were determined using a ^{125}I radioimmunoassay (RIA) kit, according to the manufacturer's instructions (MP Biomedicals).

ChIP-sequencing

To assess whole-genome hippocampal binding of pCREB and GR during the post-learning consolidation period, we used chromatin immunoprecipitation (ChIP) followed by sequencing. ChIP was performed as described before (24). Protease and phosphatase inhibitors (Roche) were added to all buffers during tissue processing and the ChIP procedure. Hippocampal hemispheres were fixated with 1% formaldehyde for 12 to 14 minutes and were homogenized in Jiang buffer using a glass douncer (Kimble-Chase). Chromatin of four hemispheres (i.e. hippocampi from two rats of the same experimental group) were pooled, resuspended in NP buffer and fragmented by sonication for 32 cycles (30 seconds ON/30 seconds OFF) using a Bioruptor (Diagenode). Tissue of rats from different training time and days were pooled to prevent an effect by (time of the) day. From each chromatin sample (A-B-C-D; n=4 biological replicates) an input aliquot was taken, which resulted in a combined input sample per treatment group (1-2-3-4; 50 µL total). Subsequently, the chromatin sample was split for a paired pCREB and GR measurement (700 µL each), using 4 µg of anti-phospho-CREB Ser133 antibody (17-10131, Millipore) or 6 µg of anti-GR antibody H-300 (sc-8992X, Santa Cruz). Background signal was detected on one of the chromatin samples with a ChIP using 6 µg of control IgG antibody (ab37415, Abcam). After several washing steps (24), antibody-bound DNA was collected with 250 µL elution buffer [0.1 M $NaHCO_3$, 1% SDS] while shaking at 37°C for 15 minutes. Input and eluted ChIP samples were decrosslinked (400 mM NaCl, overnight at 65°C), purified by phenolization and pellets were dissolved in 60 µL TE buffer [10 mM Tris-HCl, 1 mM EDTA]. Of this, 10 µL was kept for qPCR validation and 50 µL was used for sequencing. qPCR was performed on 6x diluted ChIP samples according to the protocol described below.

Before sequencing, adapters (Agilent) were ligated and samples (4 input and 4x4 ChIP samples) were subjected to 15 rounds of PCR for DNA library preparation (KAPA Biosystems). Single-end sequencing was performed on a HiSeq 2500 (Illumina) at High Output. Due to overrepresentation of the input samples, the ChIP samples were sequenced over two runs to obtain the intended number of reads. In the first run 51 bp

were sequenced; as a result of developments at the sequencing facility (The Netherlands Cancer Institute) this was increased to 65 bp for the second run. Combined, the two runs gave a total of 13.5-24.8 million reads per pCREB ChIP sample and 11.0-22.5 million reads per GR ChIP sample.

Peak calling and differential binding analysis

For read quality control, read alignment and peak calling the Carp pipeline v0.8.0, published as part of Bio Pipeline Execution Toolkit (Biopet), was used. Biopet contains the main sequencing analysis pipelines developed at Leiden University Medical Center with code being accessible at https://github.com/biopet/biopet. The rest of the analysis was done using custom scripts developed for this particular project.

Reads were aligned to *Rattus norvegicus* genome version 6 (rn6) with short read aligner bwa-mem version 0.7.10. Peaks were called using Model-based Analysis of ChIP-Seq (MACS2) version 2.1.1.20160309 (25), invoking subcommand "callpeak". Used MACS2 settings were: effective genome size = 2.00e+09; q-value cutoff = 0.05; bdg = true. For every sample, an input sample (one per treatment group) was provided. For both pCREB and GR, this step provided 16 (4 replicates for each of the 4 treatment groups) BED files with peak (narrowPeak) locations in each sample.

Separately for pCREB and GR, the corresponding 16 BED files were merged using mergeBed version 2.26.0, resulting in a list per TF with locations of all peaks found in any of the treatment groups. Overlapping peak regions were replaced by unions of the regions, leading to a single regions BED file for pCREB and one for GR. For the calculated regions and for each sample read counts were generated using htseq-count v0.6.1p1. Tool settings used were: -s no, -m intersection-strict, -f bam.

For the differential binding analysis, we selected only regions which were present in a minimum of 3 out of 4 replicates for at least one of the treatment groups. The goal of the analysis was to find treatment effects on binding of each of the TFs. We used the TMM method (26) to normalize for library sizes and the edgeR method (27) for identification of regions with differential counts. For the pCREB dataset, two of the samples (group 3 replicate B and group 4 replicate A) were identified as outliers and excluded from further analysis. Four contrasts between the treatments were studied (Differential group 2 versus group 1 (D21), D31, D42, D43; according to the group designations in **Figure 2A**). We used a 0.05 threshold on FDR to classify a peak region as bound differentially between treatments. Peak files were annotated using HOMER with *Rattus Norvegicus* v6.0.89 gtf file.

Motif analysis

Sequences of the most robust GR peaks, those present in at least 3 out of 4 replicates, were examined for enrichment of TF motif occurrence. We used MEME (Multiple Em for Motif Elicitation) for *de novo* motif analysis and MAST (Motif Alignment and Search Tool) to search specifically for glucocorticoid response elements (GREs) (28), as reported previously (24).

Table 1. Primer sequences used for qPCR on rat hippocampal ChIP samples (DNA) and cDNA (intronic primers to measure pre-mRNA).

Target	Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
ChIP DNA	*cFos*	Fos proto-oncogene, AP-1 transcription factor subunit	GGGGCGTAGAGTTGATGACA GCAATCGCGGTTGGAGTAGT	152
	Per1	Period circadian regulator 1	GGAGGCGCCAAGGCTGAGTG CGGCCAGCGCACTAGGGAAC	73
Pre-mRNA	*Fkbp5*	FK506 binding protein 5	GCAACCTCGAGGACTTGTCA ATCAGGGCACAGTAAACGCA	105
	Gjb6	Gap junction protein, beta 6	ACACCTTTATCACGGGCGTT AAGCAAGTCTCAACCACCCC	71
	Nsmf	NMDA receptor synapto-nuclear signaling and neuronal migration factor	GCTTCTTATGAACAGCCGCC TAACGGCCATGACTGAGTGG	194
	Rplp0	Ribosomal protein lateral stalk subunit P0	GCCTGGAATTGGCAACTAAGC CAGCGGCCTGACCTTAACAT	150

Real-time quantitative PCR

Rat hippocampal hemispheres were homogenized in TriPure (Roche) by shaking the tissue with 1.0-mm-diameter glass beads at 6.5 m/s for 20 seconds in a FastPrep-24 5G instrument (MP Biomedicals). Total RNA was isolated and 4 µg of each sample was DNAse (Promega) treated for 30 minutes at 37 °C. Subsequently, cDNA was generated and RT-qPCR was performed as described before (24). Pre-mRNA was measured using intronic primers. Genes of interest were normalized against housekeeping gene *Rplp0*. Primers for qPCR on ChIP samples were designed to span the pCREB or GR binding site of positive control loci. All primer sequences are listed in **Table 1**.

Statistics

For the discrimination index one sample *t*-tests were performed to detect differences from chance level (zero) for each treatment group; one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests was used for comparison of CORT groups with the vehicle-treated animals. CORT levels in the ChIP-seq animals and pre-mRNA measurements for transcriptional validation were analyzed by two-way ANOVA with Sidak's post-hoc tests, using training status and post-training drug treatment as between-group parameters. ChIP-qPCR data were examined by two-way ANOVA per TF without follow-up tests. All data are presented as mean ± standard error of the mean.

Results

First of all, an OLM experiment was performed with different doses of CORT in order to find the optimal memory-enhancing dose (**Figure 1**). Rats were given 3 minutes of training to explore the experimental apparatus containing two identical objects and directly afterwards CORT was administered subcutaneously. Retention testing was performed 24 hours later in the same box with one of the objects placed in a new location. The discrimination index (DI), representing the level of preference for the object in the novel location, was used as a measure of memory. We tested a range of 0.3-1.0-3.0 mg/kg of CORT, along control animals that received an injection of vehicle only.

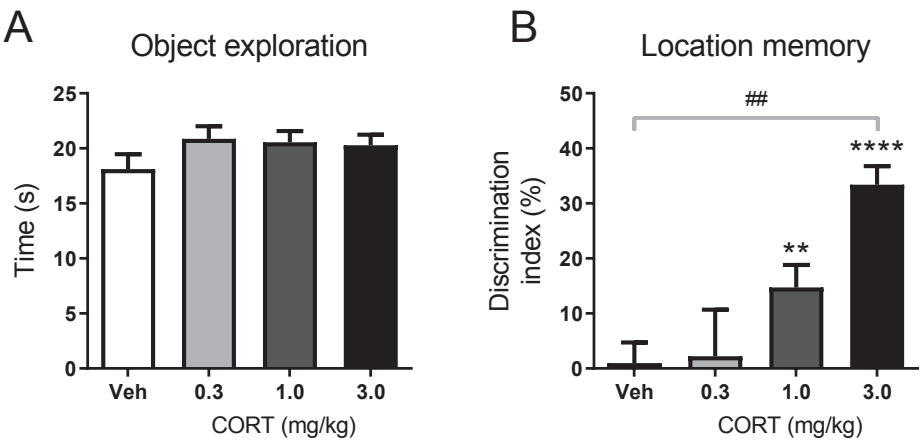


Figure 1. Object location memory. **A)** Total object exploration time of the two identical objects during the training trial and **B)** a dose-response effect of CORT on the discrimination index at a 24-hour retention test (n=8-10). CORT = corticosterone, Veh = vehicle; ** $P<0.01$, **** $P<0.0001$ compared to zero, ## $P<0.01$ compared to vehicle group

The total object exploration time during training was similar for all groups (**Figure 1A**), which indicates no differences in acquisition between the groups before the rats were injected. While rats that received vehicle or 0.3 mg/kg CORT did not exceed chance level (discrimination index (DI) of zero, indicating a 50/50 exploration of the object in the novel/familiar location), both 1.0 mg/kg and 3.0 mg/kg CORT-injected groups showed a preference for the object placed in the novel location (**Figure 1B**). Only the rats dosed at 3.0 mg/kg CORT had a significantly higher DI than the vehicle group (P = 0.0012). Subsequent OLM experiments therefore involved an injection of 3.0 mg/kg CORT for memory induction.

We proceeded with the ChIP-sequencing experiment, set up in a two-way design (**Figure 2A**). Rats had either no training or were exposed to OLM training, which induces CREB activation during the learning process (8). Directly after training the rats received either a vehicle or a CORT injection, with the latter activating GR. Our hypothesis was that pCREB and GR interact at the DNA level during the post-learning consolidation period, and a combined activation of CREB and GR (group 4) would lead to differential binding of the two TFs compared to either CREB (group 3) or GR (group 2) activation alone. A time point of 45 minutes after training was chosen to enable detection of both pCREB and GR DNA binding in the consolidation phase (29, 30).

The groups subjected to OLM training (**Figure 2B**) showed comparable total object exploration times as observed in previous experiments (**Figure 1A**), with no difference between the two groups. Elevated plasma CORT levels were confirmed in the CORT-injected animals (**Figure 2C**). We examined TF binding in whole hippocampi of all treatment groups, with group 4 representing the emotional memory formation context. ChIP was performed on pooled hippocampal tissue of two animals from the same treatment group, leading to n=4 samples for paired detection of pCREB and GR. As positive controls for the detection of TF binding, ChIP-qPCR confirmed pCREB binding at the *cFos* promoter (**Figure 3A**) and GR binding at the *Per1* promoter (**Figure 3B**), two well-known target loci for pCREB and GR, respectively (30, 31). No main effects of OLM training or CORT injection were observed for any of the TF at these two binding sites.

Next, all ChIP samples were sequenced, reads were processed and peaks were called (**Table 2**). In total 51,997 unique pCREB peaks and 30,726 unique GR peaks were detected across all samples. Merging all peaks per TF into a single BED file resulted in a median peak width of 272 bp for pCREB and 219 bp for GR. We first examined the overlap between the four biological replicates. For both pCREB and GR the majority of peaks were observed in

A

Learning (CREB activation)	GR activation: Vehicle	GR activation: CORT
No training	1	2
OLM training	3	4

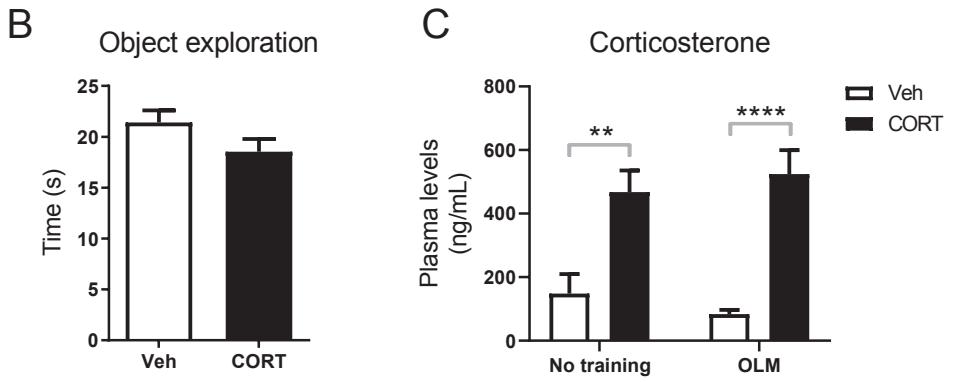


Figure 2. ChIP-sequencing for pCREB and GR. **A)** Experimental setup. **B)** Total object exploration time during training for OLM groups and **C)** plasma CORT levels of all groups at sacrifice, 45 minutes after injection (n=8). ChIP = chromatin immunoprecipitation, CORT = corticosterone, pCREB = phosphorylated cAMP response element-binding protein, GR = glucocorticoid receptor, OLM = object location memory, Veh = vehicle; ** $P < 0.01$, **** $P < 0.0001$

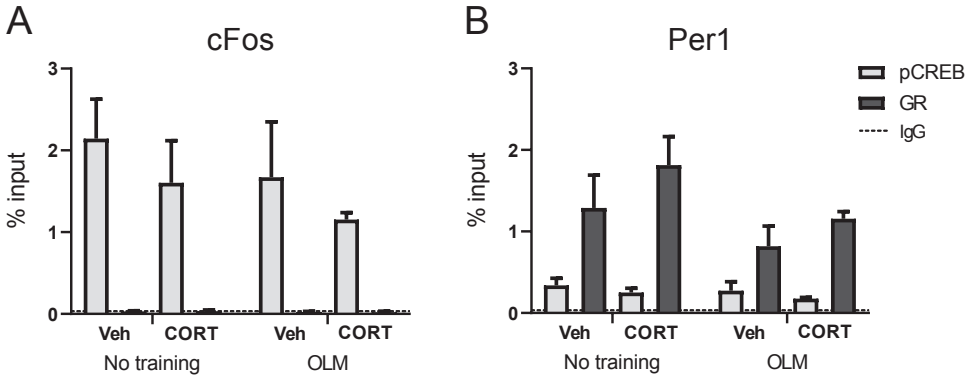


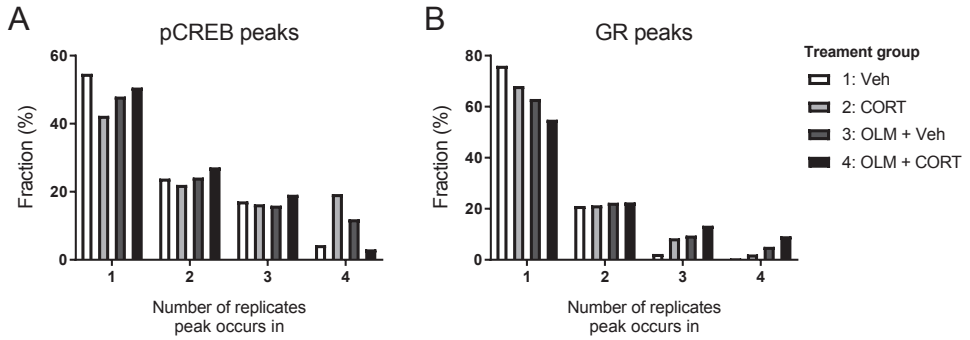
Figure 3. Positive control ChIP-qPCR. **A)** pCREB binding at the *cFos* promoter and **B)** GR binding at the *Per1* promoter as positive controls (n=4). CORT = corticosterone, GR = glucocorticoid receptor, IgG = immunoglobulin G background control, OLM = object location memory, pCREB = phosphorylated cAMP response element-binding protein, Veh = vehicle

only one of the replicates (**Figure 4A-B**). From a biological perspective, bona fide binding events affected by training and/or CORT injection would be expected to occur in multiple animals. Interestingly, the fraction of GR peaks that were observed in 3/4 or 4/4 replicates increased upon interventions in group 2-3-4 compared to the basal condition in group 1 (**Figure 4B**). We took the increased number of GR binding sites after CORT observed amongst the 3/4 and 4/4 peaks as evidence for bona fide DNA occupancy at these loci. This selection resulted in 1885 GR peaks after CORT treatment alone, while more than a double number of loci (4498) were GR-bound in the combined treatment group (**Figure 4C**), providing a first indication of an interaction between training and CORT-induced transcriptional effects. For pCREB the fraction of most robust peaks, those present in 4/4 replicates, increased with either CORT injection or OLM training, but lowered again with the combined treatment in group 4 (**Figure 4A**). Further data analysis was performed on the selection of biologically relevant peaks, i.e. those that were present in a minimum of 3/4 replicates for at least one of the treatment groups: 14,722 peaks for pCREB and 5,307 peaks for GR.

Table 2. Number of unique pCREB and GR peaks detected per treatment group.

Treatment group	pCREB peaks (#)	GR peaks (#)
1: Veh	25651	11148
2: CORT	31125	17832
3: OLM + Veh	34084	11969
4: OLM + CORT	32209	19894

To get a grasp on the type of binding sites in the ChIP-seq experiment, we conducted motif analysis on the GR peaks that were observed in 3/4 or 4/4 of the replicates. *De novo* motif analysis with MEME was however impeded by the presence of repeat regions (data not shown). Therefore, we performed a directed search for the glucocorticoid response element (GRE), the GR binding motif, using MAST. GREs were present in peaks of all treatment groups, although OLM-trained rats showed lower fractions of GRE-containing peaks than non-trained rats (**Figure 4C**). The overall increase in the fraction of robust GR-bound loci in the OLM groups relative to their corresponding non-trained group (**Figure 4B**), accompanied with a decrease of GREs found within those peaks (**Figure 4C**) is supportive for the hypothesis that the initial arousal-induced signaling pathways affect subsequent GR transcriptional activity.



C

GRE-containing peak fraction

	Vehicle	CORT
No training	**16.9%** (319)	**11.7%** (1885)
OLM training	**4.2%** (1746)	**5.2%** (4498)

Figure 4. ChIP-seq peak results. Prevalence of peaks within the n=4 biological replicates for **A)** pCREB and **B)** GR. **C)** The fraction of GR peaks containing a GRE, analyzed using Motif Alignment and Search Tool on the combined 3/4 and 4/4 peaks detected per group, with the total number of GR peaks between brackets. CORT = corticosterone, GR = glucocorticoid receptor, GRE = glucocorticoid response element, OLM = object location memory, pCREB = phosphorylated cAMP response element-binding protein, Veh = vehicle

Our main objective was to determine changes in TF binding in the different conditions. Peaks were analyzed for differential binding by a method based on RNA-sequencing analysis (**Figure 5**), in which we used pair-wise comparisons between the four different treatment groups (**Figure 2A**). For pCREB, this analysis resulted in only 6 differentially occupied peaks as a result of OLM training in both vehicle-injected (D31; 3 up) and CORT-injected (D42; 3 up) animals (**Figure 5A**). For GR, 67 of the peaks showed differential binding, mainly in response to CORT injection in non-trained (D21; 7 down and 40 up) as well as OLM-trained (D43; 1 down and 38 up) animals (**Figure 5B**). Of these differentially bound GR sites, 20 changes in binding were shared between the two CORT groups (**Figure 5C**). While OLM training itself minimally induced differential binding by pCREB or GR, independent of CORT status (D31 and D42 in **Figure 5A, 5B**), the training process did affect GR binding as the subset of loci differentially occupied upon CORT treatment also contained unique peaks for both OLM-trained (27 loci) and non-trained (19 loci) rats (**Figure 5C**).

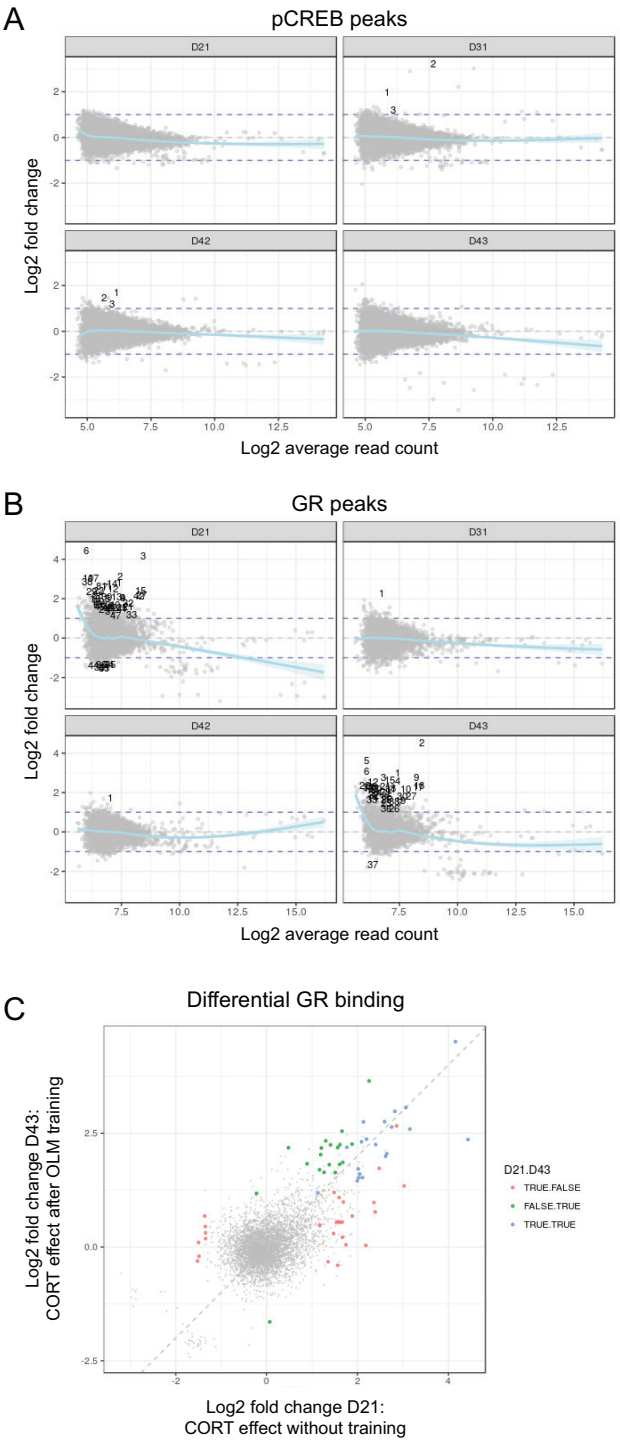


Figure 5. ChIP-seq differential binding analysis of **A)** pCREB peaks and **B)** GR peaks. Fold change of the read counts plotted against the average read count per peak, for each pair-wise comparison. Statistically differentially bound sites are indicated by black numbers. **C)** Fold changes of D21 versus D43 to visualize the overlap in differentially bound GR sites. Green data points indicate peaks affected in D21 only (TRUE.FALSE), red data points indicate peaks affected in D43 only (TRUE.FALSE) and blue data points indicate peaks affected in both contrasts (TRUE.TRUE). D21 = group 2 versus group 1, effect of CORT in non-trained animals; D31 = group 3 versus group 1, effect of OLM training in vehicle-injected animals; D42 = group 4 versus group 2, effect of OLM training in CORT-injected animals; D43 = group 4 versus group 3, effect of CORT in OLM-trained animals. CORT = corticosterone, GR = glucocorticoid receptor, OLM = object location memory, pCREB = phosphorylated cAMP response element-binding protein

This chapter focuses on further analysis of the GR dataset. Differential GR peaks after CORT treatment were filtered by annotation for intragenic or proximal promoter (up to -5kb) localization in the genome, and ranked by the highest fold change in either of the contrasts. A top 10 of the differentially bound GR sites and associated genes is presented in **Table 3**. Two of these sites were localized in adjacent introns of the gene *Engulfment and cell motility 1* (*Elmo1*). In **Table 4** the genes are listed that are associated with CORT-modulated differential peaks specifically in non-trained animals (D21), specifically in OLM-trained animals (D43), and those common for both contrasts. Noteworthy is the GR binding site near *cFos*, which is induced by CORT only in OLM-trained animals. This locus is about 8.5 kb upstream from the pCREB positive control site measured by ChIP-qPCR (**Figure 3A**). Strikingly, the single differentially bound site which shows increased occupancy upon OLM training in CORT-treated rats (D42), is a peak near *Small Nucleolar RNA 24* (*SNORA24*) that had decreased occupancy upon CORT injection in non-trained animals (D21) (**Figure 5B**). This suggest a CORT-induced downregulation of GR binding that is restored in the context of the OLM task. It might be of interest to follow up *Steroid 5 alpha-reductase 1* (*Srd5a1*), whose last intron contains the *SNORA24* coding region, and test if the close by modulated GR binding site can affect expression of this gene. Examples of ChIP-seq aligned reads are visualized for differential GR peaks near the *Fkbp5*, *Gjb6* and *Pnpla7; Nsmf* loci (**Figure 6A**).

Table 3. Top 10 differential GR binding results.

Differential peak	Annotation	Distance from TSS (bp)	Associated gene	Log2 fold change D21	Log2 fold change D43
D43_2	Intron	2864	*Aspa*	4.2	4.5
D21_6	Promoter-TSS	-176	*Gjb6*	4.4	2.4
D43_5	Intron	46910	*Elmo1*	ns	3.6
D21_2	Intron	95029	*Ntrk2*	3.2	2.6
D43_6	Intron	21730	*Plcl1*	3.1	3.1
D21_17	Intron	-39614*	*Elmo1; SNORA17**	3.0	ns
D21_14	Intron	60262	*Ptprr*	2.8	2.6
D43_9	Intron	19915	*Fkbp5*	2.1	2.7
D21_11	Intron	-10315*	*Pnpla7; Nsmf**	2.6	2.1
D43_12	Intron	47712	*Phactr3*	ns	2.5

D21 = group 2 versus group 1, effect of CORT in non-trained animals; D43 = group 4 versus group 3, effect of CORT in OLM-trained animals. For peaks with multiple associated genes, the upper gene indicates the genomic localization (annotation), although the *lower gene has the closest TSS. CORT = corticosterone, GR = glucocorticoid receptor, ns = not significant, OLM = object location memory, TSS = transcription start site

Table 4. Genes associated with CORT-induced differential GR binding.

Non-trained animals		Common	OLM-trained animals	
D21 increased	D21 decreased	D21&D43 increased	D43 increased	D43 decreased
Abhd11	*Cdkn3*	*Aspa*	*AABR07035835.1*	*Dchs2*
Armc12	*Col11a1*	*Car12*	*Adamts9*	
Cacna2d3; 5S_rRNA	*Ecd*	*Dusp1*	*Capn9*	
Elmo1; SNORA17	*Pex14; Casz1*	*Fkbp5*	*cFos*	
Farp1	*Prox1*	*Gjb6*	*Elmo1*	
Il11ra1	*SNORA24; Srd5a1**	*Gramd3*	*Fgf2*	
Lmod1	*Usp46*	*Hdgfl1*	*Gadd45g*	
Mblac2		*Hif3a*	*Grifin*	
Mgst2		*Il1rap*	*Hrh1*	
Mical2; Micalcl		*Ntrk2*	*Htra1*	
Nav3		*Olig1*	*LOC108351737*	
Pcsk2; Bfsp1		*Plcl1*	*Nxn; Mrm3*	
RGD1307100		*Pnpla7; Nsmf*	*Oacyl*	
Sec14l1; 7SK snRNA		*Ppp2r2a*	*Phactr3*	
Tex2		*Ptprr*	*RGD1566085*	
Tspan9		*Serp2*	*Sorbs1; Pdlim1*	
Ttyh1		*Slc30a5*	*U6 snRNA*	
Usp2		*Stox2*	*Xxylt1*	
Zmynd8		*Usp24*		
Znf740		*Zfp648*		

In case of two listed genes these represent: Gene of genomic annotation; gene with closest transcription start site, except for: #The *SNORA24* coding region lies within the last exon of *Srd5a1*. It should be noted that for these lists the loci specific to one of the contrasts did not reach significance for differential binding in the other contrast, but may however contain false negatives that should be listed under the common differential binding instead. CORT = corticosterone, GR = glucocorticoid receptor, OLM = object location memory

Finally, we examined hippocampal gene expression of differentially bound GR loci in an independent batch of rats exposed to the same conditions as in the original ChIP-seq experiment. We were able to validate CORT responsiveness on pre-mRNA levels in the three visualized differentially bound GR target genes: the classical and robust GR target *Fkbp5*, and the novel target genes *Gjb6* and *Nsmf* (**Figure 6B**). For all genes a main CORT injection effect was observed ($F_{1,22}$ = 94.34, P < 0.0001 for *Fkbp5*; $F_{1,19}$ = 4.409, P = 0.0493 for *Gjb6*; $F_{1,18}$ = 12.27, P = 0.0025 for *Nsmf*). *Fkbp5* expression was increased by CORT treatment in the animals that had no training, as well as in the animals that had undergone OLM training (both P < 0.0001). Elevated pre-mRNA levels of *Gjb6* were observed upon CORT

treatment specifically in non-trained animals (*P* = 0.0273), while levels of *Nsmf* increased in response to an injection of CORT in OLM-trained animals only (*P* = 0.0135). For *Gjb6* these transcriptional effects were consistent with the fold change in GR binding signal, which was higher in the non-trained animals (**Table 3**).

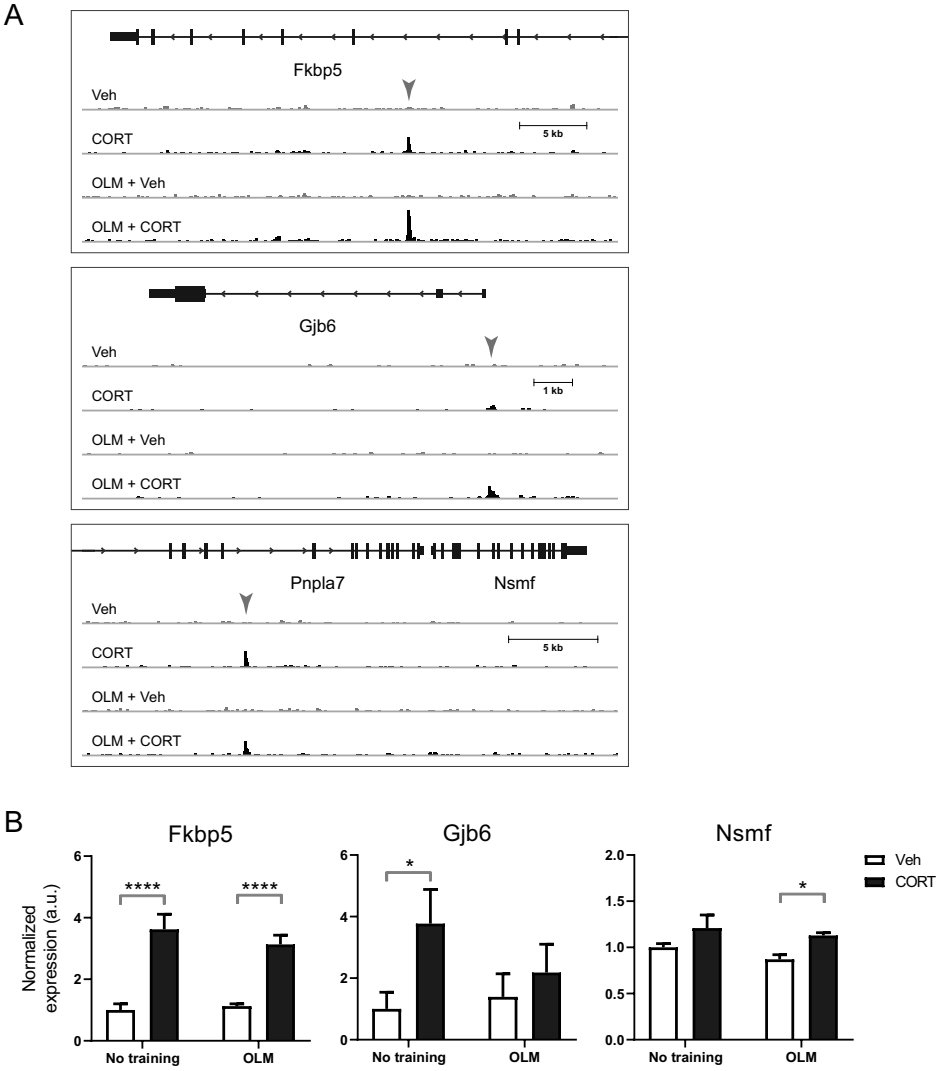


Figure 6. GR binding sites of interest. **A)** Genome browser tracks of GR occupancy at the *Fkbp5*, *Gjb6* and *Pnpla7*; *Nsmf* associated loci, visualized by one representative (replicate C) per treatment group. Note that these traces are not normalized for the amount of reads per sample. The differential peak is indicated with an arrow. **B)** Validation of transcriptional effects on pre-mRNA levels, for the genes *Fkbp5*, *Gjb6* and *Nsmf* (n=6-7). a.u. = arbitrary unit, CORT = corticosterone, OLM = object location memory, Veh = vehicle; * *P* < 0.05, **** *P* < 0.0001

Discussion

The current experiments aimed at identifying interactions between pCREB and GR that underlie emotional enhancement of memory. Using an OLM task, in which we first established the optimal memory-enhancing dose of 3.0 mg/kg CORT, combined with ChIP-seq, we detected whole-genome hippocampal DNA binding of the two TFs during the memory consolidation process. Four different treatment groups were included: no training or OLM training, followed by a vehicle or CORT injection. The OLM-trained CORT-injected animals represent the emotional learning condition, in which (nor)adrenaline and glucocorticoids together activate each of their downstream targets, pCREB and GR respectively. While a limited number of changes in pCREB binding were observed between the different conditions, GR peaks showed increased binding for 58 loci and lowered binding for 8 loci upon CORT treatment. Genes associated with differentially bound GR peaks were followed up at the pre-mRNA level, confirming regulation of classical GR target *Fkbp5* and revealing two novel GR targets, *Gjb6* and *Nsmf*.

In earlier ORM studies, a peripheral dose of 1.0 mg/kg of CORT was found to give the optimal memory-enhancing effect (8). The OLM experiments described here pointed towards 3.0 mg/kg of CORT as resulting in the most pronounced object preference. While ORM is dependent on cortical regions such as the perirhinal and insular cortex (32), OLM relies mainly on activity within the hippocampus (13). Possibly these brain regions, in combination with the type of information that has to be processed (i.e. a new type of object versus a new location of a familiar object), have a different sensitivity towards CORT and might require distinct levels of the hormone to have the same effect. Though, a recent study applying local administration into the prelimbic cortex showed that ORM might require a higher dose of a specific GR agonist than OLM in order to reach optimal memory enhancement (33). In any case, whereas endogenous CORT levels of vehicle-injected rats were not sufficient to induce memory in our OLM setup, a post-training injection of 3.0 mg/kg CORT functioned as a solid switch for memory formation.

Genome-wide binding of pCREB and GR was examined, of which we hypothesized to find loci with an effect on TF binding levels by OLM training or CORT administration alone, which is different upon exposure to both training and CORT. Several studies in various tissues suggest transcriptional interactions between (p)CREB and GR, either direct or indirect. The two TFs could cooperate in activating hepatic *glucose-6-phosphatase* expression via the shared coactivator CRTC2, reciprocally facilitating DNA binding to the required CREB response element (CRE) and GRE (34). Positive crosstalk has also been

shown at the *phosphoenolpyruvate carboxykinase* gene, and the authors demonstrated physical interaction between CREB and GR by co-immunoprecipitation (21). Furthermore, during fasting CORT levels rise and GR may enhance glucagon-induced pCREB binding in mouse liver by inducing additional pCREB binding sites as well as increasing its binding intensity (35). In contrast, GR is able to block CREB-mediated activation of *glycoprotein hormone alpha-subunit* in placental cells (36) and pCREB and GR mutually interfered with each other's binding at the *thyrotropin releasing hormone* promoter in hypothalamic neurons (37).

The here described analysis did not show many training-induced effects on hippocampal pCREB binding, and the ChIP-seq dataset needs to be analyzed further to explore anticipated pCREB-GR interactions in a memory relevant context. However, focusing on the GR binding data, the results did provide evidence of an interplay between the learning process and stimulation with CORT. The current experiment was performed in adrenally intact animals, explaining the relatively small amount of differential GR binding sites upon CORT treatment when compared to other studies with adrenalectomized animals (30, 38). Alike CORT administration, also OLM training could induce more robust GR binding sites. The lower fraction of GR peaks containing a GRE observed upon OLM training, suggests that animals undergoing memory consolidation might present with different types of GR binding that go beyond the dimeric DNA binding mode that was mainly observed in pharmacological, out-of-context GR activation (30). These may include tethering to other TFs, e.g. AP-1, FOX and STAT, and binding to negative GREs or GRE half sites (22, 38-41). In addition, OLM-trained animals showed a unique subset of differentially bound GR peaks compared to non-trained animals. Further sequence analysis might point out characteristics of the common versus the training status-dependent differentially occupied binding sites, such as GRE content and the presence of a CRE and/or binding motifs for other TFs. Though, *de novo* motif analysis gave no useful results as the GR binding sites were frequently located near simple repeats. Occupancy of these regions might be related to the fact that hippocampal GR activation is accompanied by silencing of transposable elements (42). Furthermore, such loci could be involved in looping of multiple GR binding sites, shown to occur between loci with direct and indirect GR binding modes (43). Remarkable are the loci with decreased GR binding in CORT-treated animals, indicating a loss of interaction of DNA-bound GR upon higher concentrations of agonist binding. This is in accordance with our previous mineralocorticoid receptor (MR) cistrome data, in which MR peaks seem to disappear upon higher CORT levels (24).

Although recent research demonstrated that rapid transcriptomic changes in the hippocampus are dependent on noradrenaline signaling rather than GR activity (44), this was not reflected in differential pCREB binding in our animals. Several reasons could explain the lack of changes in pCREB binding. First of all, we might have used a suboptimal timing for detection of altered pCREB occupancy. In our ChIP-seq experimental setup we aimed at detecting pCREB and GR in the same animals, at the same post-injection time point of 45 minutes. However, if changes in pCREB and GR binding do not occur simultaneously, this could imply that chromatin remodeling effects rather than direct protein-protein interactions underlie any DNA level interplay between pCREB and GR. Other studies assessed pCREB binding at earlier time points of 15-30 minutes after a stressful event (45, 46). A second hurdle was the multiple testing issue that arose with the vast amount of data generated by ChIP-seq. For pCREB almost three times the amount of GR peaks were analyzed on differential binding. Moreover, high basal binding could impede the opportunity to detect any increase of pCREB upon stress. Accordingly, the more stressful forced swim task was unable to enhance (p)CREB binding at two baseline occupied immediate early gene promoters *cFos* and *Early growth response 1* (45). In our control group, the vehicle injection could also have affected TF binding compared to naïve animals, although any transcriptional changes that might have been induced by the arousal associated with the injection procedure were not sufficient to induce memory without the administration of CORT. We also cannot exclude the possibility that changes in pCREB upon OLM training are mediated in a brain region other than the hippocampus, such as the basolateral amygdala (8), and might have an indirect effect on CORT-induced memory. Finally, it could be possible that not (p)CREB itself is affected, but downstream changes occur during OLM training that allow constitutively bound (p)CREB to have an effect, such as enabling of chromatin accessibility or unblocking of transcriptional elongation (18, 47). Supporting this notion, phosphorylation of CREB is not crucial for its role in hippocampal learning as shown in CREB Ser133 mutant mice (48). Besides, CORT-induced memory in the OLM task has been demonstrated to be dependent on the interaction of pCREB with CREB-binding protein (13), a coactivator that also cooperates with GR signaling (49).

The newly identified GR targets *Gjb6* and *Nsmf* provide relevant starting points for further mechanistic investigation of convergence of the (nor)adrenaline and CORT pathways. While the classical GR target *Fkbp5* showed increased expression upon CORT treatment independent of training status, the induction was specific to non-trained rats for *Gjb6* and to a lesser extent to OLM-trained rats for *Nsmf*. Encoded by the *Gjb6* gene, Connnexin 30

creates astrocytic gap junctions and can restrict the survival of adult newborn neurons (50). *Gjb6* has been reported previously as a glucocorticoid-responsive gene in the mouse cortex and rat hippocampus (51), but had not been proven to be a direct GR target. We could speculate that out-of-context GR activation induces *Gjb6* expression to restrict the formation of new neurons, while in the case of stimulating GR in a learning context this blockage of neurogenesis is relieved. The *Nsmf* gene encodes the protein Jacob, a messenger involved in the transmission of NMDA receptor signaling to the nucleus, where it is believed to interact with the CREB transcriptional complex (52). Our findings indicate that GR can induce the expression of *Nsmf*, which in turn can link the activity-dependent NMDA receptor signals during learning to CREB-dependent gene expression, posing an indirect interaction between CREB and GR signaling. *Nsmf* knockout mice show impaired contextual (i.e. hippocampal dependent) fear condition and OLM performance (53). Of note, the neurons of these mice also presented with decreased basal pCREB levels. Given that inducible *Nmsf* knockout mice are available, it will be interesting to test the hypothesis that OLM performance cannot be enhanced by CORT treatment in these animals.

In our ChIP-seq dataset we expected to find a limited number of differentially bound pCREB and GR loci associated with regulation of genes that can link the emotional experience and memory enhancement. The current analysis focused on the GR data and confirmed hippocampal *Fkbp5, Gjb6* and *Nsmf* upregulation in response to CORT treatment. It would be of interest to examine other brain regions to find out about specificity of these targets in animals subjected to the same task as well as other behavioral paradigms. The nature of pCREB-GR interactions should be further delineated using transgenic mice with defined deficiencies in CREB or GR signaling, combining behavioral tasks and DNA occupancy to identify molecular mechanisms and additional candidate loci that are crucial for stress-potentiation of learning. A priori it is not clear whether the GR target genes responsible for memory consolidation should be induced only after OLM – functional analysis of validated target genes will have to tell this in future.

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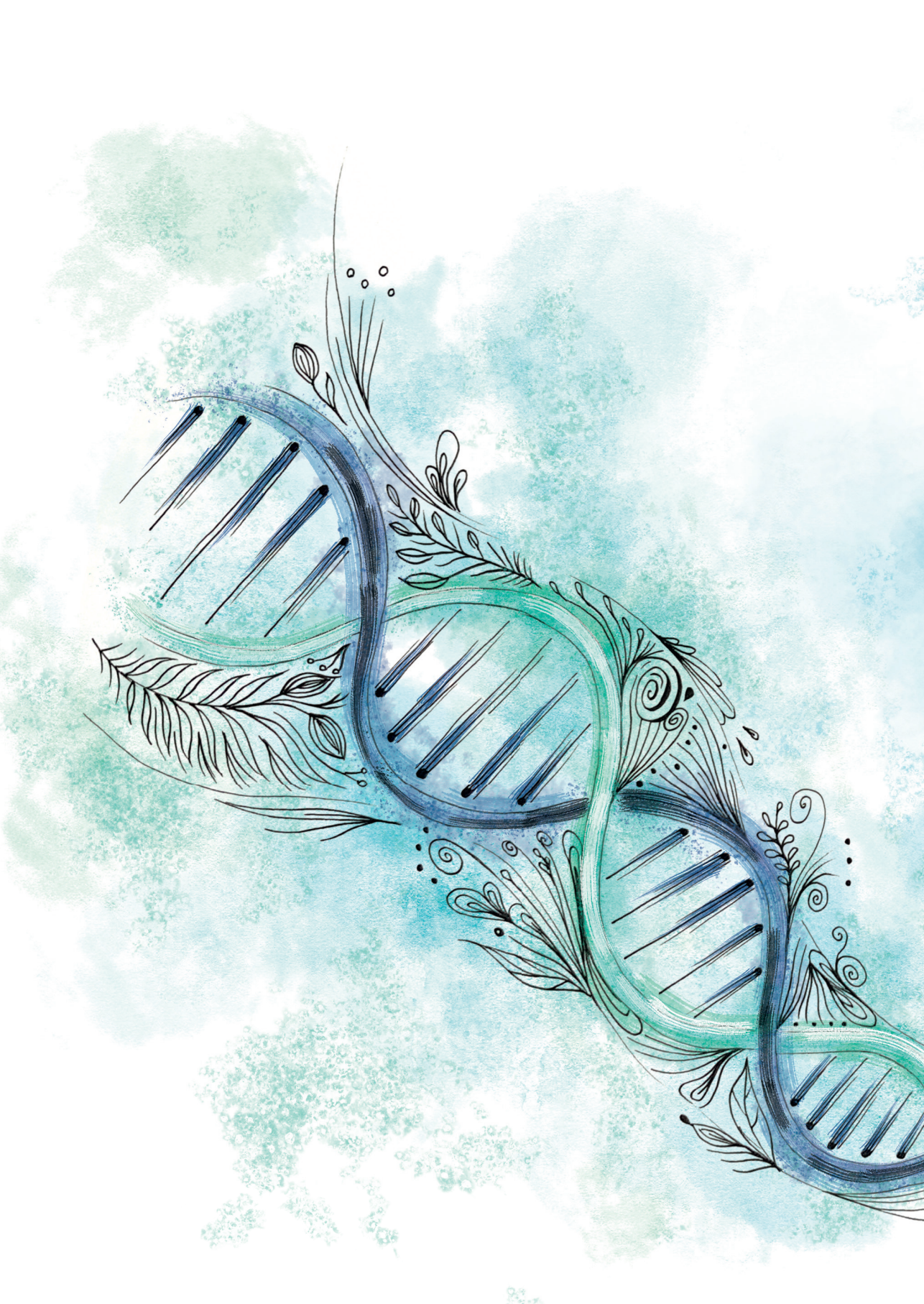

CHAPTER 6

Summary and general discussion

More than 45 years of research on the effects of glucocorticoids on brain function has yielded many insights (as outlined in the introduction), but also left a number of long-standing questions. One conundrum has been how activation of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) can lead to very different, or even opposite effects. It also remained unclear how the consequence of activation of a single receptor, GR, can differ from cell to cell and from situation to situation. A mechanistic basis for appropriate changes in gene expression that underlie the adaptive effects of stress steroids is the diversity of MR/GR signaling partners, involving coregulatory proteins and other, non-receptor transcription factors (TFs). In this thesis we have investigated two specific aspects of transcriptional regulation in response to glucocorticoids in the brain: the cause of MR/GR specificity, and the role of crosstalk with other TFs. This final chapter will summarize novel insights from the in this thesis described studies, followed by general discussion of the data, functional and clinical significance and future perspectives.

Summary

The first research chapters (**Chapter 2, 3 and 4**) examined the genomic interactions of MR compared to GR, and the common and specific transcriptional responses mediated by the two receptor types. In **Chapter 2** we used chromatin-immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) to obtain hippocampal genome-wide DNA binding profiles for MR and GR. This was done in brain tissue of adrenalectomized rats that had received an intraperitoneal injection of corticosterone 60 minutes prior to sacrifice. Comparison of MR and GR cistromes resulted in 918 MR-exclusive sites, 1450 GR-exclusive sites and another 475 MR-GR overlapping sites. Of note, the MR binding sites were detected for two different dosages of corticosterone (0.3 mg/kg and 3.0 mg/kg) and, in contrast to our expectations, limited overlap was found between MR cistromes upon the lower and higher hormone concentration. We validated several MR-exclusive target loci by ChIP-qPCR in an independent set of adrenally intact animals, around the time of their endogenous corticosterone peak. Since DNA binding by MR/GR needs consecutive modulation of gene activity to eventually have functional consequences for a (brain) cell, we studied associated transcriptional effects in **Chapter 3**. In order to filter out false positive putative targets, we focused on binding sites that were located within gene bodies or the (proximal) promoter region. Subsets of MR-specific, GR-specific and MR-GR overlapping targets were assessed in a forebrain MR knockout model (fbMRKO). In these mice, a decreased expression was found for a number of predicted MR-specific targets, for the classical glucocorticoid target gene *Fkbp5* and a couple of other overlapping targets, and – surprisingly – for two predicted GR-specific target genes. The most robust effect was observed on mRNA levels of the MR-specific target *Jdp2*. This was (besides the panel of classical targets) the sole MR/GR target that was responsive (i.e. upregulated) in subsequent validation using the model of restraint stress. We thus identified *Jdp2* as a bona fide hippocampal MR-specific target gene.

In the studies described in **Chapter 2** we also examined sequences of the DNA fragments defined by the MR and GR peaks. Virtually all sites bound by MR and/or GR contributed to *de novo* detection of the glucocorticoid response element (GRE). In addition, we were surprised to find that all MR-exclusive sites were associated with an Atoh1 consensus site (part of the group of 'E-box sequences'), which was not retrieved from the GR-exclusive or MR-GR overlapping dataset. Based on their hippocampal expression, we hypothesized NeuroD family members to bind this additional sequence. Using ChIP-qPCR, we could indeed confirm *in vivo* Neurod2 occupancy near MR-exclusive loci. Next, we studied the NeuroD proteins that are expressed in adulthood (Neurod1, Neurod2 and Neurod6)

in reporter assays driven by a promoter that contained a GRE with an adjacent Atoh1 site (GRE-At). These experiments were performed in HEK293 cells, to which expression plasmids for the receptors had to be added as well. All three NeuroD family members were able to potentiate corticosterone-induced transactivation at this construct, for both MR- and, unexpectedly, GR-transfected cells. This effect was not dependent on either the N-terminal or C-terminal part of MR/GR, as demonstrated by the use of truncated versions of the receptors. We explained the *in vitro* lack of specificity for potentiation of MR over GR signaling to be likely a result of the absence of a neuronal-specific chromatin/cellular context, and formed the novel hypothesis that additional factors mediate an indirect effect of NeuroD on glucocorticoid signaling. In **Chapter 4** we aimed to further explore the mechanism behind the NeuroD-mediated enhancement of MR signaling. We first demonstrated by ChIP-qPCR in fbMRKO animals that Neurod2 binding was independent of MR binding. Also GR binding was unaffected by the absence of MR for the target loci tested, except for a slight increase of GR occupancy at the *Per1* promoter. The purpose of following experiments was to find out which part of the NeuroD protein is responsible for its potentiation of glucocorticoid signaling. Various NeuroD-related E-box binders (MyoD, Myf5 and a MyoD truncation) were studied in our (adapted) GRE-At reporter assay. MyoD was able to potentiate MR/GR transactivation when its DNA binding domain was replaced with that of Neurod2, or when the E-box sequence in the luciferase promoter was adjusted to be effectively bound by MyoD. This latter construct was further studied in combination with the several E-box binders. We showed that MyoD variants harboring their domain responsible for chromatin remodeling activity, but lacking an activation function for direct recruitment of the transcriptional machinery, could still enhance MR/GR-mediated transcription. Our overall conclusion was that NeuroD acts permissively to enable MR binding rather than prevent GR binding, and chromatin remodeling seems the main mechanism driving NeuroD potentiation of MR signaling.

The interaction between GR and other TFs has mainly been studied in cell line models. In **Chapter 5** we examined GR context-dependency at a genome-wide scale *in vivo*, in a memory-relevant behavioral model. To this end, we made use of an object location memory (OLM) task in which glucocorticoids can act as a switch for long-term memory formation, but this is dependent on training-induced noradrenergic signaling. One of the TFs activated (i.e. phosphorylated) by noradrenaline is cAMP response element-binding protein (CREB). We therefore assessed the potential interaction of GR with pCREB. In our setup, vehicle-injected animals did not discriminate between objects. Corticosterone-injected animals (3.0 mg/kg, subcutaneous) on the other hand, showed

evident preference for the object in a new location relative to that in the familiar location, serving as a measure of memory. Four treatment groups were examined for DNA binding of the two factors: [1] non-trained vehicle-injected control animals, [2] non-trained corticosterone-injected animals to observe the effect of GR activation, [3] OLM-trained vehicle-injected animals to observe arousal-induced changes in pCREB, and [4] OLM-trained corticosterone-injected animals to observe the effect of combined CREB and GR activation. In each of these groups genome-wide binding of pCREB and GR within the hippocampus, at a timepoint of 45 minutes after the injection, was measured by ChIP-seq. We included the most robust peaks (i.e. those present in 3/4 or 4/4 of the biological replicates) in our analysis. Interestingly, the GRE content of the GR peaks detected in OLM-trained animals was lower compared to the non-trained groups, suggesting that the mode of GR signaling is affected by the training status. Peaks were analyzed for changes between treatment groups. As few as 6 loci were found differentially occupied by pCREB and we decided to focus on the GR binding data in the analysis. Amongst the GR peaks, we found 67 differentially occupied loci, mainly in response to corticosterone treatment. Of these, 20 loci were affected independent of training status, while 27 loci were specific to non-trained animals and 19 loci specific to OLM-trained animals. We subsequently confirmed corticosterone-mediated gene expression changes on pre-mRNA level for the classical target gene *Fkbp5*, as well as newly identified GR targets *Gjb6* and *Nsmf*. Overall, we provided evidence that the GR cistrome, whether or not as a result of interactions with pCREB, can be affected by exposure to a training task.

Towards an updated corticosterone receptor model

1. MR-mediated effects in the higher corticosterone range

Back in 1985 it was shown by Reul and de Kloet that MRs and GRs are differentially distributed in the brain, but colocalized in hippocampal neurons. They also demonstrated that corticosterone has a tenfold higher affinity for the MR than for the GR (1). Since then, we have had the view that MR is occupied by hormone under basal conditions and GR gets bound in conditions with elevated hormone levels. The general assumption has therefore also been that corticosterone concentrations that exceed 'basal hormone levels' lead to GR-mediated effects. In other words: the GR is the receptor for stress-induced increases in corticosterone (2). Later work showed that non-genomic effects mediated by hippocampal MRs require higher corticosterone concentrations (3), but the notion of the 'saturated MR' has held for its genomic effects.

In **Chapter 2** of this thesis we however showed that two high doses of corticosterone which ought to be both super-saturating for MR, could still lead to differences in target gene binding. We observed other binding sites in response to 'very high' 3.0 mg/kg corticosterone compared to 'high' 0.3 mg/kg corticosterone (at which receptors should already be saturated). Unexpectedly, an increased hormone concentration was thus able to induce binding of MRs to additional sites. Apparently not only non-genomic but also genomic MR is sensitive for hormone changes in the stress-range. For novel target *Jdp2* its promoter binding by MR was demonstrated in **Chapter 3** to be accompanied by stress-responsive regulation of the gene.

A possible explanation for the discrepancy between hormone concentrations needed for maximal occupancy of MR and those leading to maximal DNA binding effects may be the transient corticosterone peak applied in our ChIP-seq experiment (4), while K_d determinations take place under steady-state conditions (5). Also the lag between hormone binding and nuclear translocation should be taken into account, as well as the subsequent step of stable DNA binding (6, 7). Finally, there may be signal integration with the lower affinity membrane receptors, which could lead to e.g. changes in MR phosphorylation that might be needed for binding to specific DNA loci (8). In any case, the MR cistrome was clearly affected by higher than 'basal' hormone concentrations, and we should adjust our MR/GR model accordingly. RNA-seq experiments using different corticosterone doses would have to reveal subsequent implications for corresponding target genes.

2. Reaching MR versus GR specificity

Upon the discovery of MR and GR presence in the hippocampus, initial functional findings pointed to complementary effects of MR and GR on behavior (9) and even opposite effects on neuronal excitability (10). However, around the same time the molecular structure of the two receptors was found to be very similar, in particular in the DNA binding domain (11). In accordance, MR and GR can both bind the GRE sequence and concomitantly transactivate genes. The receptors do strongly differ in their capacity to interact with other TFs, e.g. in case of transrepression of AP-1 and NF-κB (12). Also, GR is uniquely capable of repressing transcription via negative GREs (13). However, in ChIP-seq data available from the hippocampus the predominant binding mode of both MR and GR is to GREs. This suggests that the opposite effects mediated via MR and GR on e.g. hippocampal CA1 cells must be caused by receptor-specific GRE-driven target genes.

In **Chapter 2** we indeed describe unique as well as shared loci for MR and GR in hippocampal chromatin. The unique sites all contained GREs in association with other motifs, which presumably bind other TFs that transfer the specificity. This was in fact predicted by K. Yamamoto who found that evolutionary conservation of the GRE predicted functionality (i.e. binding) of that sequence (14). He noted that a receptor molecule does not 'know' whether a sequence is conserved in the DNA of another species. Therefore, the conservation of the 15-nucleotide long GRE likely reflects a larger stretch of DNA which includes binding sites for other TFs, and these confer the actual capacity for receptor binding, and perhaps specificity. This model is supported in a previous study on the hippocampus in which binding sites for SP-1 family members distinguished functional from non-functional GREs with respect to GR binding (15).

The additional motifs associated with GR-exclusive binding in our study were left unexplored, though some potential cross-talk partners were detected in a distinct subset analysis (16). However, for the Atoh/NeuroD motif that we found in all of the MR-exclusive binding sites, we showed actual binding of Neurod2 to these loci. This finding puts MR in a longer list of nuclear receptors that – in a tissue-specific manner – rely on additional TF presence for their binding and/or functionality. For example, mouse liver GRs depend on bHLH protein E47 at many loci (17) and estrogen receptors interact with pioneering factor FoxA1 (18). The NeuroD-MR link is in all likelihood specific for brain MR, but in other MR-expressing tissues similar proteins may provide context specificity at the chromatin level. Our work described in **Chapter 2** suggests that there are additional proteins involved in the specific interaction between MR and NeuroD factors, as in reporter assays in non-neuronal cells also GR activity was enhanced by NeuroD proteins.

Experimental follow-up on the NeuroD-MR link puts challenges. First, there are several NeuroD family members, which all might interact with MR. Second, it is difficult to recapitulate the cellular context of end-differentiated cells, even in iPSC-derived cultures. Knockout of NeuroD factors will interfere with neuronal differentiation (19-21), and this may also happen when NeuroD is inactivated in end-differentiated neurons (given their continued presence). We therefore, in the work described in **Chapters 2 and 4**, used more simple systems with controlled expression of the various factors, and made use of closely related MyoD proteins that bind related E-box sequences. These experiments brought us insights on potential mechanisms of interaction. We could differentiate between domains necessary for chromatin remodeling and direct transactivation, and we have shown that the interaction likely involves addition proteins. In this respect it would be interesting to compare MR and GR complexes with RIME methodology (22), in which

a comprehensive characterization of interacting proteins is achieved. Another attractive option is to study MR and GR interactions with other TFs using proximity ligation assays (23). These approaches should serve to confirm receptor-specific protein interactions, and determine the extent of brain region, cell-type, context, and species specificity of the findings reported in this thesis.

Intriguingly NeuroD is involved in the differentiation of particular neuronal phenotypes, which apparently includes MR function. Even though MR may respond to corticosterone levels in the stress range, as shown in this thesis, its affinity for MR is tenfold higher than for GR. As a result, it sets the sensitivity of the hippocampal circuitry for activation, with consequences for both cognition and mood. Genetic as well as human pharmacological data suggest that a gain-of-function variant of the MR confers resilience to depression, in particular in premenopausal women (24, 25). Current data on potential functional interactions between MR and NeuroD factors are scarce. Elevated Neurod2 levels were found in the ventromedial prefrontal cortex of depressed *men* (26) – it is for now unclear how these factors relate, considering that postmortem studies often have many experimental issues, including the use of medication. Even though, the finding would be consistent with one study suggesting that in males the more active variant of MR increases the risk for depression (27). Neurod2 has been found to be significantly co-expressed with the 5-HT1A receptor in the human brain (28), which is one of the signaling pathways that was controlled by MR in the original studies on rodent hippocampus (29). However, no genetic associations between NeuroD proteins and mood disorders have been discovered to date.

Next to establishing a possible mechanistic basis for MR-specific effects, our data also provide leads to the actual genes and proteins that underlie such effects. Considerable efforts have gone into candidate gene approaches to understand, for example, genes and proteins that drive modulation of CA1 pyramidal neuron excitability (30). Our bottom-up approach of identifying MR-specific loci, and linking these to gene expression resulted in a number of – likely – bona fide MR-specific target genes in the mouse brain. We established for a small number of genes that they were clearly expressed at lower levels in the brains of fbMRKO mice. Combining our ChIP-seq with RNA-seq will in future likely reveal more MR-specific target genes. *Jdp2* mRNA also responded to stress-induced corticosterone elevations, which may be an example of MR-mediated functional effects at concentrations traditionally considered as 'super-saturating'. While we did not formally prove that these genes are not regulated via GR, it will be interesting to evaluate their expression in particular settings. One such setting is exposure to high levels of synthetic

GR-selective glucocorticoids. The suppressed cortisol that accompanies this kind of treatment is predicted to deprive the MR of its ligand. This may actually contribute to the psychiatric side effects of treatment with GR-selective drugs (31, 32), and lower expression of MR target genes would substantiate this notion. The MR-specific target genes that we found may be used in further studies in the context of mood regulation.

3. Binding at MR-GR joint sites

While MR-specific target genes may explain some of the intrinsic genomic MR-mediated effects that are unique to this receptor, it has long been clear that MR and GR have a very similar DNA binding domain, and that they can bind to identical GRE sequences. In fact, canonical GR target genes such as *Gilz* and *Sgk1* were independently characterized as functionally important MR target genes (33, 34). We confirmed that MR binds to the *Fkbp5* gene and observed that *Fkbp5* expression was reduced in the hippocampus of fbMRKO mice. This finding seems quite relevant to those studying the effects of chronic stress on the brain. *Fkbp5* expression is routinely used as a readout for GR activation (35). The protein Fkbp5 is part of the complex that regulates ligand binding and nuclear translocation of GR, and its upregulation by GR provides intracellular negative feedback. It has been proposed to be a mediator of long-term stress effects in the brain (36), in part via methylation of its promoter (37, 38), and Fkbp5 inhibitors are considered for clinical development in psychiatry (39, 40). Our data call for a reevaluation of MR in these effects, including the notion that Fkbp5 may also act as a co-chaperone for factors other than GR.

The regulation of genes via both MR and GR would expand the effective concentration range of corticosterone for these genes by an order of magnitude. From the overlapping binding sites in our ChIP-seq dataset we are not able to tell if these are derived from a combination of MR and GR homodimers binding the same locus in different neurons, or that MR-GR heterodimers (41) were present in our samples. However, using re-ChIP in the hippocampus at particular loci the *in vivo* formation of heterodimers has been made plausible (42). Heterodimerization may also explain why others found that a subset (15%) of hippocampal GR DNA binding sites was also associated with NeuroD factors (43). These likely represent MR-GR overlapping target loci as described in **Chapter 2**, at two of which in **Chapter 4** we have detected Neurod2 binding as well. Besides binding of each heterodimer partner to a half-site of the GRE, co-occupancy of GREs by MR and GR could also be realized via higher order complexes (44), or with MR tethering to GR (6). The DNA occupancy studies do not allow to unequivocally determine whether the outcome of such MR-GR interactions is additive, synergistic, or rather antagonistic. Although hippocampal

GR is upregulated in fbMRKO mice, this apparently could not compensate for the lack of MR transcriptional activity at the several overlapping genes that were downregulated in these animals. It will be interesting to see in functional studies whether the receptors cooperate, counteract or simply have independent effects.

GR interactions during memory consolidation

Since the 1980s we are well aware that GR via corticosterone influences the process of memory consolidation (9, 45, 46), which was later shown to be mediated by transcriptional responses of the receptor (47). In fact, in the setup that we used in **Chapter 5**, corticosterone can act as a switch for long-term memory consolidation. Because administration of beta-blockers prevents the effect of corticosterone (48), we hypothesized that there is a molecular interaction between two downstream effectors of noradrenaline and corticosterone, pCREB and GR respectively. We found limited evidence for such an interaction on the DNA level. For GR binding we did observe a mild context-dependency, while for pCREB differences between groups were almost absent. Future gene expression studies – at multiple timepoints after corticosterone treatment – should determine whether the transcriptional outcome of GR activation is also context dependent.

We worked under the assumption that pCREB and GR would act as a genomic 'coincidence detector' within hippocampal neurons. However, since we assessed whole hippocampi, we cannot exclude dilution effects. *Arc* reporter mice show a clear mosaic activation of neurons after learning experiences, and only in those cells CREB seemed activated (49). Therefore, single cell approaches (50) may yield outcomes that are more in line with our original hypothesis, and show more context dependent changes with respect to pCREB, as well as GR binding. Of course, our hypothesis may also be wrong. The potentiation of learning could alternatively involve noradrenaline-induced GR modification. Other studies showed reduced coimmunoprecipitation of CREB with a GR phosphorylation site mutant (51) and a unique gene regulatory profile of specific GR phospho-isoforms (52). Moreover, because the brain is a network there is the possibility that noradrenaline and glucocorticoids independently affect different neuronal populations, e.g. in amygdala and hippocampus (48). We must conclude that despite the elegance of our behavioral setup with corticosterone as memory switch, our study did not resolve the question of how GR acts differently at the genome in order to facilitate memory consolidation.

Nevertheless, the different experimental conditions tested in the ChIP-seq study of **Chapter 5** might provide us with greater understanding of several psychopathologies. The strengthening of memory consolidation by stress is considered part of the pathogenesis of post-traumatic stress disorder by many (53, 54). Transcriptional changes that depend on co-activation of CREB and GR are of particular interest to this situation. On the other hand, high levels of glucocorticoids per se (i.e. stress without a particular learning context) may be relevant to any stress-related psychopathology, although typically chronic rather than transient exposure is looked at.

Technical considerations and future approaches

The work in this thesis generated new insights, but of course there is much more to do. It is a truism that the design of the experiment determines the outcome. In this light, there seems value in reiterating some aspects of the here described studies. Our DNA binding data were obtained within an hour of corticosterone treatment, but in two very different conditions. In **Chapter 5** we saw that a relatively mild contextual change of training versus control may already affect GR binding. In **Chapter 2** we studied the cistromes of MR and GR in adrenalectomized rats which were at rest. Another recent study did not find any differences in GR binding upon restraint stress compared to similar corticosterone exposure in a non-stressed control situation (43), and the type of stressor as well as the lack of adrenals in those animals might have had a role in that negative finding. Therefore, it will be crucial to tailor future work to specific physiological or pathological contexts. It also needs to be kept in mind that DNA binding does not equal transcriptional activity (55) and in many cases MR/GR occupancy might hold a permissive effect on gene expression rather than having a strong regulatory role on its own. Furthermore, to predict MR-regulated target genes, we have limited ourselves to loci within or very close to genes. Techniques that map the three-dimensional conformation of the genome, such as 4C and Hi-C (56), will support a more careful annotation of binding events and can reveal long-range interactions of loci that affect sites of transcriptional activity further than their nearest gene (4). Even if we were able to identify additional unknown target genes for the MR based on ChIP-seq data, it will be good to combine future ChIP-seq studies with measurements on genomic spatial organization as well as transcriptomics to directly link DNA occupancy to functional binding events.

DNA binding that is associated with transcriptional changes, still brings the issue of timing. For example, in **Chapter 5** we evaluated pCREB binding 45 minutes after training,

and we may have missed transient effects given that changes in transmitter activity occur almost instantaneously (57). On the other hand, we evaluated gene expression by looking at unprocessed transcripts (pre-mRNA) at the same time point, and this in all likelihood is too early to detect many changes. Also time of the day is a relevant factor, as the expression of or occupancy by signaling partners may show circadian variation (58). Ideally, time courses would be constructed both for GR DNA binding and transcriptional responses, but given the budgets necessary for omics studies, the considerations remain difficult when addressing the effects of transiently changing hormone levels.

Since the start of the work described in this thesis there has been an impressive development and implementation of new techniques to assess transcriptional effects and chromatin regulation. Some of these can also be applied *in vivo*. ATAC-seq is one such an approach (59), which may be used to gauge the overall accessibility of chromatin, as a consequence of MR or GR activation. Given the presence of particularly GR in many different neuronal and non-neuronal cells in the hippocampus, it is advisable to reduce cellular complexity before applying such technology. At the proteomics level, RIME is a promising technique (22) to assess proteins that are in the same complex as the receptors, and this may be used to confirm and expand data on other TFs that interact with MR and GR to establish their cell- and context specific effects. Furthermore ChIP-exo has an increased resolution compared to traditional ChIP assays, as the binding site is narrowed down to physically protected bases. However, this comes with the disadvantage of more challenging data analysis because of e.g. increased amount of multiple reads to be mapped to the same locus (60).

Once MR/GR loci and predicted target genes are identified, a next challenge is to determine the contribution of individual genes and proteins to hippocampal functioning. This challenge amounts to creating shortlists from longlists. Combining primary targets (ChIP-seq) with transcriptome data provides a filter, but additional strategies seem necessary to pinpoint targets that can be functionally studied using knockout and knockdown models. Of course, for lack of true shortlists, a biologically informed hypothesis and the availability of mouse models can lead to meaningful results. In this respect it would be interesting to for example test whether mice that lack *Nsmf* (61) display potentiation of memory formation after GR activation.

While the work described in this thesis addressed basic questions, there are immediate applications for clinical research. The established MR target genes may not only be helpful in the context of dexamethasone-induced psychiatric side effects, but also in relation to

chronic exposure to endogenous glucocorticoids during chronic stress and in Cushing's disease. They may also be useful to evaluate the effects of hyperaldosteronism as occurs in Conn's syndrome. Those patients also report psychological disturbances, and these may well involve MR target genes in the brain, either in the aldosterone-selective brain stem neurons or via classical cortisol-preferring MRs as present in the hippocampus. Given that MR gain-of-function seems to protect against affective disorders, the MR-dependent cistrome (and transcriptome) should hold cues to factors that confer resilience to stress-related disorders.

Concluding remarks

We have shown that the dogma of MR saturation and its function being restricted to basal hormone levels is incorrect, since increasing corticosterone does yield additional genomic MR binding. We identified NeuroD as the factor driving MR over GR binding specificity in the hippocampus. Finally, we have explored context dependency of GR genomic action in a model that uses corticosterone as a switch for memory consolidation. More experiments are needed in which hormone effects are determined in relevant experimental settings, such as behavioral tasks related to learning and memory. Combining these with ever expanding databases on the genome, and tissue-specific expression of signaling partners, should speed up our understanding of the role of MR- and GR-dependent signaling in relevant adaptive and pathophysiological settings over the coming years.

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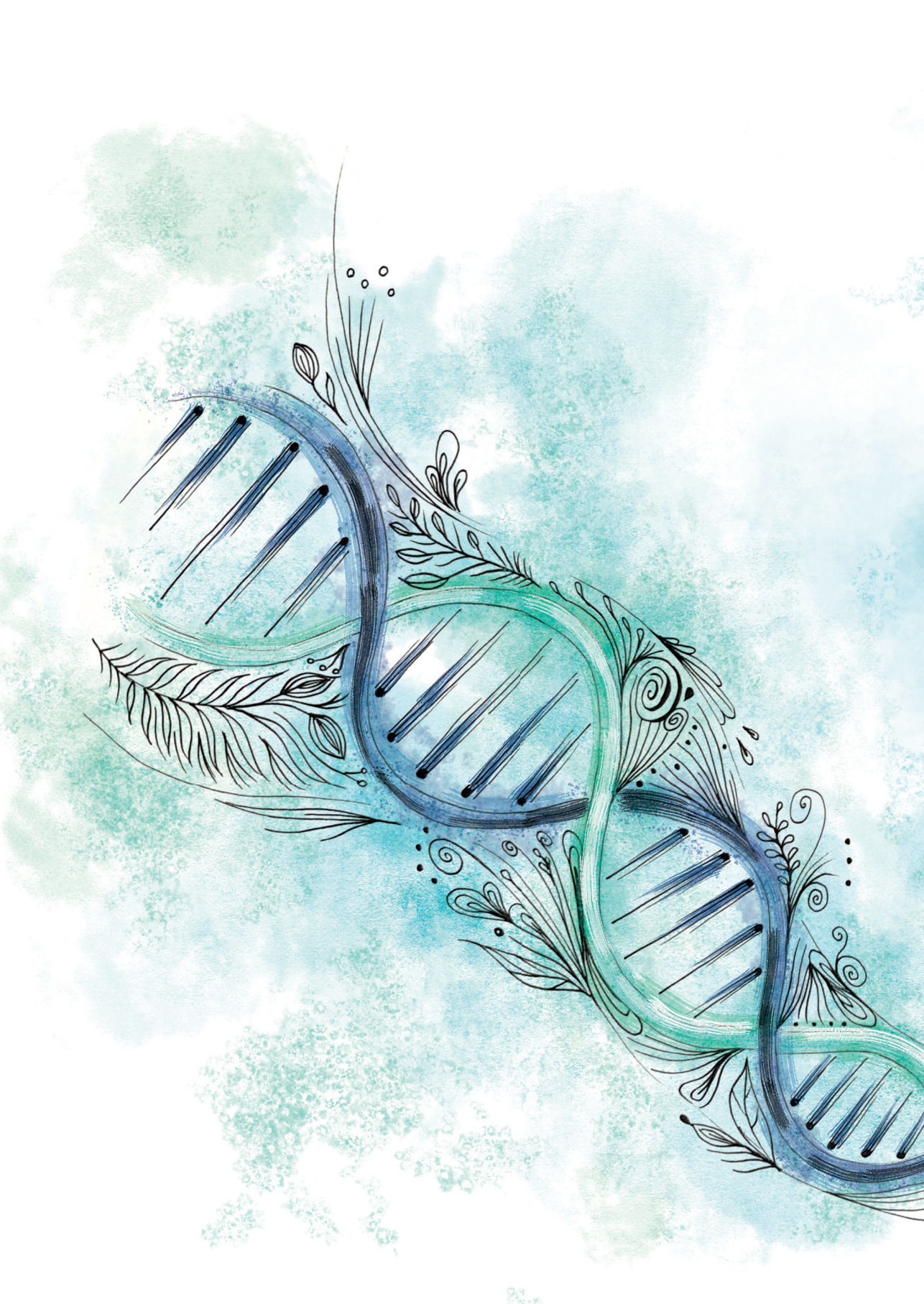

CHAPTER 7

Nederlandse samenvatting
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Nederlandse samenvatting

Als gevolg van stress worden stresshormonen aangemaakt in de bijnieren. Allereerst komt het snelwerkende adrenaline vrij, waarvan noradrenaline het equivalent is in de hersenen. Daarnaast worden met enige vertraging glucocorticoïden (corticosteron in knaagdieren en cortisol in de mens) afgegeven. De glucocorticoïd hormonen activeren twee vergelijkbare receptoren: de mineralocorticoïd receptor (MR) en de glucocorticoïd receptor (GR). Ondanks dat de MR en GR qua structuur op elkaar lijken, hebben ze interessant genoeg een verschillende en soms zelfs tegengestelde functie. Zo is de MR meer betrokken bij het begin van de stressrespons, namelijk de inschatting van een situatie en hoe daarmee wordt omgegaan. De GR speelt daarentegen een rol in de herstelfase, waaronder het verwerken en vastleggen van een stressvolle gebeurtenis in het geheugen. Veel van de effecten die MR en GR teweegbrengen, worden in gang gezet via het reguleren van genexpressie: de receptoren binden op het DNA en oefenen zo invloed uit op de mate van transcriptie van genen. Het is voor deze genomische effecten lange tijd niet bekend geweest in hoeverre bindingsplekken op het DNA overlappen voor MR en GR. Gezien beide receptoren in de hippocampus voorkomen en daar van cognitief belang zijn, leent dit hersengebied zich uitstekend voor het onderzoeken van zowel de MR en GR samen, als de GR in de context van een geheugentaak. In dit proefschrift zijn twee aspecten van de glucocorticoïd transcriptiebiologie bestudeerd: de manier waarop MR/GR-specificiteit wordt bereikt, en de rol van interactie met andere transcriptiefactoren (TFs).

De eerste onderzoekshoofdstukken (**Hoofdstuk 2, 3 en 4**) bestuderen de genomische interacties van MR ten opzichte van GR, en de gemeenschappelijke en specifieke transcriptionele effecten die door de twee receptor typen worden bewerkstelligd. In **Hoofdstuk 2** hebben we chromatine-immunoprecipitatie (ChIP) gevolgd door sequencen (ChIP-seq) uitgevoerd, om genoom-wijde DNA-binding profielen voor MR en GR te bepalen. Dit werd gedaan in hippocampusweefsel van bijnierloze ratten die 60 minuten voor opoffering een corticosteron injectie toegediend hadden gekregen. Vergelijking van de MR- en GR-bindingsplekken resulteerde in 918 MR-exclusieve, 1450 GR-exclusieve en 475 MR-GR overlappende locaties op het DNA. Bepaling van de MR-bindingsplekken is gebaseerd op twee verschillende doses (0.3 mg/kg en 3.0 mg/kg) en er werd, in tegenstelling tot onze verwachtingen, beperkte overlap gevonden tussen de MR-bindingsplekken na toediening van de lagere versus hogere hoeveelheid hormoon. Met een ChIP-qPCR meting konden een aantal MR-exclusieve bindingsplekken worden bevestigd in een onafhankelijke groep van bijnier intacte dieren, ten tijde van hun

lichaamseigen corticosteron piek. Aangezien DNA-binding door MR/GR vervolgens ook tot modulatie van genexpressie moet leiden om consequenties te kunnen hebben op het functioneren van een (hersen)cel, hebben we in **Hoofdstuk 3** gekeken naar geassocieerde transcriptionele effecten. Hierbij hebben we gefocust op bindingsplekken die zich in of dichtbij een gen (promotor regio) bevonden. Een selectie van MR-specifieke, GR-specifieke en MR-GR overlappende potentiële targets werden bestudeerd in een voorhersenen MR knockout (fbMRKO) model. In deze muizen werd een lagere expressie gevonden voor een aantal voorspelde MR-specifieke targets, voor het klassieke glucocorticoïd target gen *Fkbp5* en een aantal andere overlappende targets, en – verassend genoeg – ook voor twee voorspelde GR-specifieke target genen. Het meest robuuste effect werd gezien op mRNA niveaus van het MR-specifieke target *Jdp2*. Dit was (naast het panel van klassieke targets) het enige MR/GR-target dat reageerde (namelijk: hoger tot expressie kwam) bij verdere metingen in een model van restraint stress. We hebben daarmee *Jdp2* geïdentificeerd als bonafide hippocampale MR-specifiek target gen.

In de studies beschreven in **Hoofdstuk 2** hebben we ook de DNA-sequenties onderliggend aan de MR- en GR-bindingsplekken geanalyseerd. Nagenoeg alle locaties gebonden door MR en/of GR droegen bij aan *de novo* detectie van het glucocorticoïd respons element (GRE). Daarnaast waren we verrast door het feit dat alle MR-exclusieve locaties werden geassocieerd met een Atoh1 consensus site (behorend bij de groep van 'E-box sequenties'), welke niet werd gedetecteerd in de GR-exclusieve of MR-GR overlappende dataset. Op basis van hun aanwezigheid in de hippocampus stelden we de hypothese dat eiwitten van de NeuroD familie aan deze extra sequentie zouden binden. Door middel van ChIP-qPCR konden we inderdaad bevestigen dat *in vivo* Neurod2 gebonden was aan het DNA in de buurt van MR-exclusieve bindingsplekken. In daaropvolgende experimenten hebben we de in volwassenheid aanwezige NeuroD eiwitten (Neurod1, Neurod2 en Neurod6) bestudeerd in reporter assays gedreven door een promotor met een GRE en naastliggende Atoh1 bindingsplek (GRE-At). Deze experimenten werden uitgevoerd in HEK293 cellen, waaraan ook expressie plasmiden voor de receptoren toegevoegd moesten worden. Alle drie de NeuroD familieleden konden corticosteron-geïnduceerde transactivatie op dit construct potentiëren, voor zowel MR- als onverwachts ook GR-getransfecteerde cellen. Dit effect was niet afhankelijk van de N-terminus of de C-terminus van de MR/GR, zoals duidelijk werd bij het gebruik van ingekorte versies van de receptoren. De *in vitro* afwezigheid van specificiteit voor potentiëren van MR- ten opzichte van GR-signalering verklaarden we door het ontbreken van een neuronale chromatine/cellulaire omgeving. Zodoende vormden we de nieuwe hypothese dat

additionele factoren betrokken zijn bij een indirect effect van NeuroD op de glucocorticoïd signalering. In **Hoofdstuk 4** hebben we het mechanisme onderliggend aan NeuroD-gemedieerde versterking van MR-signalering verder onderzocht. Allereerst lieten we door middel van ChIP-qPCR in fbMRKO dieren zien dat Neurod2-binding onafhankelijk was van MR-binding. Ook GR-binding werd niet beïnvloed door afwezigheid van MR voor de bestudeerde bindingsplekken, behalve een licht verhoogde GR-bezetting op de *Per1* promotor. Het doel van vervolgexperimenten was om te achterhalen welk deel van het NeuroD eiwit verantwoordelijk is voor het potentiëren van glucocorticoïd signalering. Verschillende NeuroD-gerelateerde E-box binders (MyoD, Myf5 en een ingekorte MyoD variant) werden bestudeerd in onze (aangepaste) GRE-At reporter assay. MyoD was in staat om MR/GR-transactivatie te potentiëren wanneer het DNA-bindings domein was vervangen door dat van Neurod2, of wanneer de E-box sequentie in de luciferase promotor was aangepast om effectief MyoD te binden. Dit laatste construct werd verder bestudeerd in combinatie met de verschillende E-box binders. We lieten zien dat MyoD varianten inclusief een domein dat verantwoordelijk is voor chromatine hermodellering, maar ontbrekend aan een activatie functie voor directe aantrekking van transcriptionele machinerie, de capaciteit behielden om MR/GR-gemedieerde transcriptie te versterken. Onze algehele conclusie was dat NeuroD de MR-binding vergemakkelijkt in plaats van dat het GR-binding voorkomt, en dat chromatine hermodellering het drijvende mechanisme lijkt te zijn voor deze NeuroD potentiëring van MR-signalering.

De interactie tussen GR en andere TFs is voornamelijk bestudeerd in cellijn modellen. In **Hoofdstuk 5** hebben we *in vivo* op genoom-wijde schaal GR context-afhankelijkheid onderzocht, in een geheugen-relevant gedragsmodel. Hiertoe hebben we gebruik gemaakt van een object locatie geheugen (OLM) taak, waarin glucocorticoïden kunnen dienen als schakelaar om lange termijn geheugenvorming te induceren. Dit effect is echter afhankelijk van training-geïnduceerde noradrenerge signalering. Een van de TFs die geactiveerd (namelijk gefosforyleerd) wordt door noradrenaline is cAMP-responselement-bindend eiwit (CREB). Daarom hebben we de potentiële interactie van GR met pCREB geëvalueerd. In onze proefopstelling maakten vehicle-geïnjecteerde dieren geen onderscheid tussen de objecten. Corticosteron-geïnjecteerde dieren (3.0 mg/kg, subcutaan) daarentegen lieten een duidelijke voorkeur zien voor het object op de nieuwe locatie ten opzichte van het object op de vertrouwde locatie, dienend als maat voor geheugen. Vier behandelgroepen werden onderzocht op DNA-binding van de twee factoren: [1] niet getrainde vehicle-geïnjecteerde controledieren, [2] niet getrainde corticosteron-geïnjecteerde dieren om het effect van GR-activatie te observeren, [3]

OLM-getrainde vehicle-geïnjecteerde dieren om veranderingen in pCREB als gevolg van alertheid te observeren, en [4] OLM-getrainde corticosteron-geïnjecteerde dieren om het effect van gecombineerde CREB- en GR-activatie te observeren. In elk van deze groepen werd genoom-wijde binding van pCREB en GR in de hippocampus, op een tijdspunt van 45 minuten na de injectie, gemeten door middel van ChIP-seq. We includeerden de meest robuuste pieken (d.w.z. welke aanwezig waren in 3/4 of 4/4 van de biologische replica's) in onze analyse. Interessant genoeg was de fractie van de gedetecteerde GR-pieken die een GRE bevatte in OLM-getrainde dieren lager ten opzichte van niet getrainde groepen, wat suggereert dat de wijze van GR-signalering wordt beïnvloed door training status. Pieken werden geanalyseerd voor verschillen tussen de behandelgroepen. Er werden slechts 6 bindingsplekken gevonden die differentieel bezet waren door pCREB. Dit heeft ons doen besluiten te focussen op de GR-binding data in de verdere analyse. Onder de GR-pieken bevonden zich 67 differentieel bezette bindingsplekken, voornamelijk als gevolg van corticosteron behandeling. Hiervan werden 20 bindingsplekken onafhankelijk van training status door het hormoon beïnvloed, terwijl 27 bindingsplekken specifiek waren voor niet getrainde dieren en 19 bindingsplekken specifiek waren voor OLM-getrainde dieren. Vervolgens bevestigden we corticosteron-gemedieerde genexpressieveranderingen op pre-mRNA niveau voor het klassieke target gen *Fkbp5*, evenals de nieuw geïdentificeerde GR-targets *Gjb6* en *Nsmf*. We hebben bewijs geleverd dat GR bindingsplekken, al dan niet als gevolg van interacties met pCREB, kunnen worden beïnvloed door blootstelling aan een trainingstaak.

List of publications

Book chapter:

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Curriculum vitae

Lisa Theodora Christina Maria van Weert werd geboren op 26 november 1989 te Schijndel. Zij groeide daar op met haar ouders en jongere broers Daan en Koen. In 2008 behaalde ze haar VWO diploma aan Gymnasium Beekvliet te Sint-Michielsgestel. Datzelfde jaar begon ze met de bacheloropleiding Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. Bij de afdeling Medical Pharmacology van het (destijds) Leiden/Amsterdam Center for Drug Research, geleid door prof.dr. Ron de Kloet, werkte ze tijdens haar bachelorstage aan het kloneren van de glucocorticoïd receptor (GR) met als doel het bestuderen van receptor-coregulator interactie profielen van verschillende GR mutanten. Onder supervisie van dr. Ioannis Zalachoras en prof.dr. Onno Meijer deed ze bij diezelfde afdeling een onderzoeksproject voor haar masteropleiding Bio-Pharmaceutical Sciences. Met antisense oligonucleotiden en door middel van een selectieve modulator onderzocht zij glucocorticoïd hormoon effecten. Tijdens haar studententijd gaf ze middelbare scholieren bijles in de bètavakken. Daarnaast werden door haar verscheidene keuzevakken gevolgd aan de Vrije Universiteit Amsterdam. Voor haar tweede masterstage verbleef Lisa een half jaar in Kopenhagen. Bij de afdeling Drug Design and Pharmacology van University of Copenhagen onderzocht zij het effect van anabole steroïden op het immuunsysteem. Na het *cum laude* behalen van haar masterdiploma werd haar de KNMP studentenprijs toegekend.

In september 2013 startte Lisa met haar promotieonderzoek naar moleculaire aspecten van stress hormoon signalering in de hersenen bij de afdeling Interne Geneeskunde, divisie Endocrinologie van het Leids Universitair Medisch Centrum. Hierin werd zij begeleid door haar promotor prof.dr. Onno Meijer, in samenwerking met co-promotor prof.dr. Benno Roozendaal van de afdeling Cognitive Neuroscience van het Donders Institute for Brain Cognition and Behaviour te Nijmegen. Gedurende haar promotietijd heeft ze studenten onderwezen bij werkgroepen, colleges, practica, het schrijven van onderzoeksvoorstellen, en begeleid tijdens wetenschappelijke stages. In 2015 heeft zij de jaarlijkse Nuclear Receptor Research Network meeting mede georganiseerd. Daarnaast heeft zij haar onderzoeksresultaten op vele (inter)nationale congressen mogen presenteren en is daarbij beloond met diverse beurzen en prijzen. Het hoogtepunt hiervan was de uitnodiging om te komen spreken op het International Aldosterone Forum te Tokyo, Japan in 2016.

Het promotieonderzoek beschreven in dit proefschrift werd afgerond in 2018. Lisa is in 2019-2020 werkzaam geweest als Scientist Biology bij het Pivot Park Screening Centre in Oss, waar ze ultra high-throughput screening projecten leidde. Begin 2021 keerde ze terug in de academische wereld, als wetenschappelijk docent bij het Universitair Medisch Centrum Utrecht. Hier is ze coördinator van de Biomedical Sciences masteropleiding 'Cancer, Stem Cells & Developmental Biology'.

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