Expression and function of nuclear receptor coregulators in brain: understanding the cell-specific effects of glucocorticoids

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Chapter I

General Introduction

1. Introduction

Based on the concept described by the French physiologist Claude Bernard (1813-1878) of 'le milieu interieur', Walter Cannon (1871-1945), a pioneering 20th century American physiologist, formulated the idea of homeostasis for living organisms. He introduced the term in the following context: "The coordinated physiological reactions which maintain most of the steady states in the body are so complex, and are so peculiar to the living organism, that it has been suggested that a specific designation for these states be employed - homeostasis" (1929). In most mammals, when homeostasis is threatened, such as in a situation of acute danger, a hormonal cascade initiating in the brain, known as the hypothalamus pituitary adrenal axis (HPA axis), is activated (fig. 1). As a result, blood glucocorticoid levels increase to support return to homeostatic set-point. More recently, in addition to homeostasis the concept of allostasis was introduced (1). Allostasis implies that in the brain adaptation to stress requires changes in structure and function of specific neural circuits to attain a new setpoint in homeostasis.

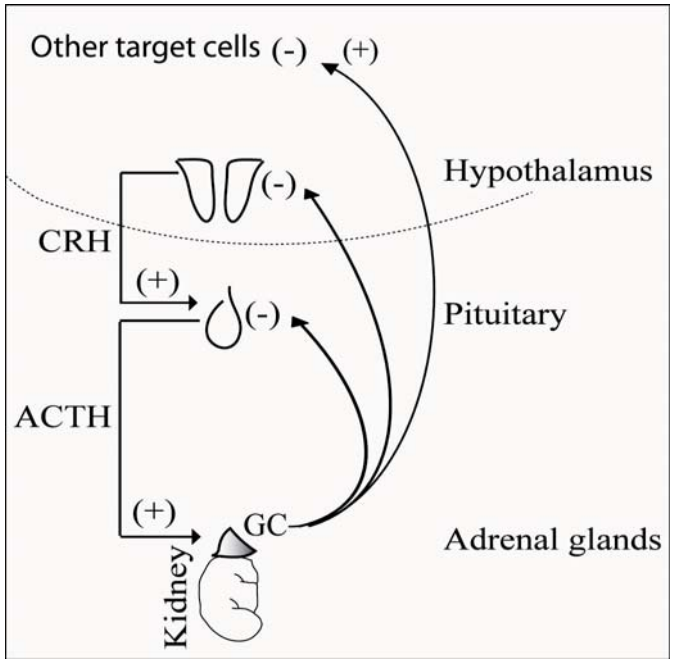

Fig.1: Representative scheme of the hypothalamus-pituitary-adrenal axis (HPA axis). Upon activation, parvocellular cells in the PVN release corticotrophin releasing hormone (CRH). CRH stimulates the expression of adrenocorticotropin hormone (ACTH) which in turn enhances glucocorticoid (GC) secretion from the adrenal cortex. Via the corticosteroid receptors in the pituitary and the PVN, glucocorticoids exert a direct negative feedback control on ACTH and CRH production.

Physiological effects of glucocorticoids take place in different time domains, and bear relevance for many aspects of the stress response, ranging from stimulatory and supportive to dampening effects in order to prepare the organism for the future (2;3). Glucocorticoids promote emotions, motivation and cognitive processes as well as energy metabolism under stress. However, stress reactions that overshoot may become damaging themselves if not controlled by glucocorticoids. The concept of glucocorticoids dampening the acute stress response of the body was developed as early as the 1950s, by Marius Tausk (4). For instance, psychological stressors evoke neurochemical reactions, which all are suppressed by

glucocorticoids in a manner reminiscent of glucocorticoid control of inflammatory reactions to tissue damage, and immune reactions to infection. This principle is the basis for the anti-inflammatory actions of synthetic glucocorticoids, such as prednisone and dexamethasone, that function to 'contain' the acute stress response (5).

The powerful effects that adrenal extracts could have on physiology were already known early in the 20th century. This work led to the purification of corticosteroids in the 1930s (6;7) followed by synthesis of cortisone in 1946 (8). The successful administration of cortisone in patients with rheumatoid arthritis triggered the development of many synthetic glucocorticoid analogues. The strong anti-inflammatory effects of dexamethasone and prednisolone (synthetic glucocorticoids) classifies them among the most successful drugs in history. However, in spite of their success, upon prolonged usage glucocorticoids have strong side effects due to their widespread actions in the body, such as for example on metabolism, bone and central nervous system (9).

A detailed understanding of the molecular mechanisms that compose glucocorticoid signaling in cells is needed in order to gain insight in how the endogenous stress response affects the vulnerability and resilience for stress-related pathology (10;11). Over the last decades an impressive amount of research has been performed combining behavioral biology, biochemistry, and, for the last 20 years, molecular biology, resulting in textbook knowledge on their mode of action. A number of important landmarks are listed in table 1. However, more work is needed to fully decipher the different mode of actions of the receptors in order to be able to develop synthetic glucocorticoids with optimized clinical properties (i.e. with fewer side effects).

Synthesis and release of glucocorticoids from the adrenals is for a large part governed by the activity of the neuroendocrine HPA axis. Upon release, glucocorticoids limit their own production by inhibiting the synthesis of the initial signaling factor of the HPA axis, *i.e.* corticotrophin-releasing hormone (CRH). They do so by suppressing CRH gene expression and secretion in the hypothalamus. This control mechanism constitutes a negative feedback loop. While CRH synthesis in the hypothalamus is suppressed, production of CRH in the central nucleus of the amygdala (CeA) is stimulated by increasing glucocorticoids levels (fig. 2).

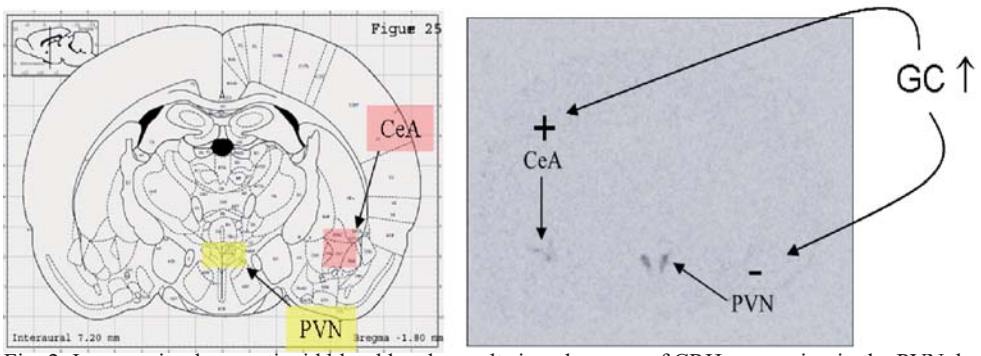

Fig. 2: Increase in glucocorticoid blood levels results in a decrease of CRH expression in the PVN, but concurrently stimulates CRH expression in the CeA in the rodent brain. The mechanism(s) by which glucocorticoids can exert cell-specific opposing effects on CRH gene expression in the rodent brain remain yet unexplained. PVN = paraventricular nucleus; CRH = corticotrophin releasing hormone; CeA = central nucleus of the amygdala; GC = glucocorticoids (see colour image page 122).

Despite two decades of intensive research the mechanisms that may account for these cell-specific effects of glucocorticoids in brain have not yet been fully understood. The work described in the current thesis is aimed at contributing to the understanding of the molecular mechanisms by which glucocorticoids can mediate such cell-specific effects in brain.

Year	Milestone
1936	Isolation of corticosteroids from bovine adrenal (Mason et al., 1936; Reichstein, 1936).
1946	Synthesis of cortisone (Sarett, 1946).
1948	First glucocorticoid treatment for Rheumatoid Arthritis (Hench et al., 1950).
1950	Nobel prize in Medicine for discoveries related to the adrenal hormones (Reichstein, Kendall and Hench).
1951	Development concept of corticosteroids as containing stress response (Tausk, 1951).
1959	Synthesis of dexamethasone.
1968	First report on selective retention of 3H-corticosterone in hippocampus (Mcewen et al., 1968).
1972	first cortisol RIA (Ruder et al., 1972).
1980	Selective agonists discriminate between the corticosteroid receptors (Moguilewsky and Raynaud, 1980).
1983	Glucocorticoid receptor acts as transcription factor through DNA binding (Chandler et al., 1983).
1985	Mineralocorticoid and glucocorticoid receptors act as a dual receptor system in brain (Reul and de Kloet, 1985).
1985	Cloning of the glucocorticoid receptor (Hollenberg et al., 1985).
1987	Cloning of the mineralocorticoid receptor (Arriza et al., 1987).
1988	11-HSD confers aldosterone specificity to the mineralocorticoid receptor (Funder et al., 1988).
1990	Transrepression by the glucocorticoid receptor via protein-protein interactions (Jonat et al., 1990).
1994	Mechanistic differentiation between transactivation and transrepression (Heck et al., 1994).
1995	mdr1a Pgp can hamper entry of dexamethasone in tissues (Schinkel et al., 1995).
1995	First steroid receptor coactivator cloned and characterized (Onate et al., 1995).
2005	Non-genomic effects mediated via classical mineralocorticoid receptor (Karst et al., 2005)

Table 1: Milestones in glucocorticoids research.

2. Corticosteroid receptors: a dual receptor system

2.1 Glucocorticoid signaling: many factors involved

Glucocorticoids are a class of steroid hormones that can bind to the same receptors and trigger similar effects. Cortisol is the most important glucocorticoid hormone in humans whereas corticosteone is the most abundant in rodents. Because glucocorticoids regulate a variety of vital physiological processes, many synthetic glucocorticoids have been designed among which dexamethasone is widely used in research because of its high affinity for the receptors.

Glucocorticoid receptor signaling firstly depends on blood levels of the hormones. There is intricate control over hormone concentration and availability by HPA axis regulation of steroid synthesis and secretion by the adrenals, secondly by binding to circulating corticosteroid binding globulin and bioconversion (12), and finally by uptake barriers at certain tissues (e.g. brain) (13;14). There are two types of receptors that can bind the main endogenous corticosteroids cortisol and corticosterone, the mineralo- and the glucocorticoid receptor

(MR and GR) (15). These receptors differ in binding affinities, tissue distribution as well as effector mechanisms, but both predominantly act as transcription factors (see below). In addition, over the last years, we have come to realize that there must be non-receptor factors that interact with the corticosteroid receptors and determine the response to glucocorticoid signaling. Among these is a group of proteins called transcriptional coregulators, which are enzymatically active proteins that bridge the DNA bound steroid receptor to the transcription machinery and act as modifiers of the chromatin structure.

2.2 Two receptor system

The mineralo- and the glucocorticoid receptors (MR and GR) belong to a superfamily of 48 nuclear receptor proteins that are critically involved in eukaryotic gene expression. Closest related to the MR and GR are the other members of the steroid receptor family ('class I') including the estrogen, the progesterone and the androgen receptors (ER, PR and AR) (16). In particular, the progesterone and androgen receptors share many structural and functional features with the corticosteroid receptors. With regard to function-structure relationships, detailed biochemical studies of partially purified receptor proteins revealed that their domain structure is highly similar within the family (17;18). Three main structural domains were described: the N-terminal region, the centrally located DNA-binding domain and the C-terminal region containing the ligand-binding pocket of the receptor.

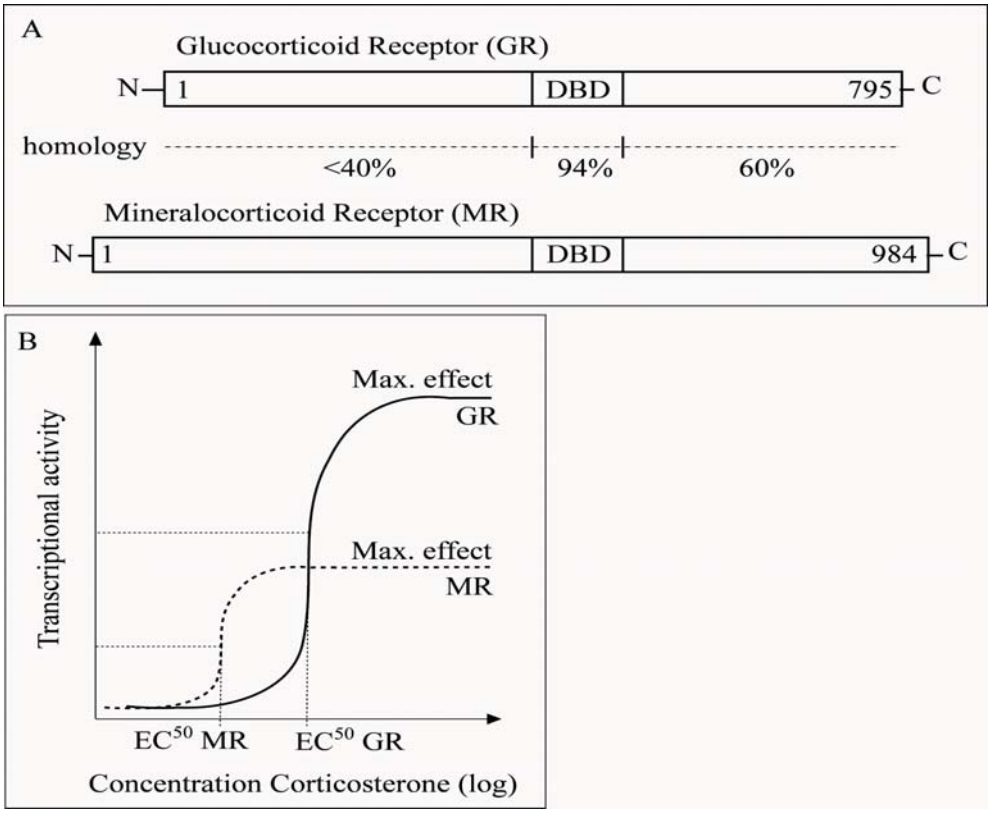

Fig. 3: (A) Schematic lay-out of the glucocorticoid and mineralocorticoid receptors (GR and MR) and homology in their amino acid sequence. Both receptors belong to the large superfamily of nuclear receptors and contain three distinctive domains: the N-terminal, the DNA-binding and C-terminal domains. (B) Dose-response curves of the GR and MR on gene transcription. While the GR has a higher transcriptional activity, the affinity of the MR for its cognate ligand is 10x higher.

In general, the agonist-bound GR has a higher transcriptional activity compared to the MR. However, the MR has a 10-fold higher affinity for corticosterone, reflected by its much lower EC_{50} concentration (Fig. 3) and therefore is thought to be substantially occupied even at basal levels of HPA axis activity (19). On the other hand, GR becomes progressively activated when corticosterone levels increase such as during stress, the ultradian hourly rhythm or at the circadian peak. MR also functions as a receptor for mineralocorticoids, such as aldosterone, most notably in kidney, where MR stimulation leads to salt retention.

The activity of glucocorticoids is tightly coupled to the action of 11beta-hydroxysteroid dehydrogenase enzymes. These enzymes catalyze the interconversion of active 11-hydroxy-glucocorticoids and their respective inactive 11-keto forms (cortisone and 11-dehydrocorticosterone) in cells. The 11beta-hydroxysteroid dehydrogenase type 1 that produces cortisol is responsible for the success of exogenous (but inactive) cortisone in the earliest clinical applications of glucocorticoids. The expression levels and the activity of these enzymes are important determinants for the bioavailability of the ligands. In aldosterone target cells, glucocorticoid levels are effectively reduced by the oxidizing type 2 form of the enzyme (12;20). In other tissues, such as liver, fat cells and brain active glucocorticoids can be regenerated locally from the inactive metabolites by type 1 (12).

2.3 Molecular mechanisms

Binding of glucocorticoids to the corticosteroid receptor leads to modulation of gene expression in the following minutes to hours. First, ligand-binding induces allosteric changes in the receptor that causes the detachment of a complex of associated proteins including chaperone proteins hsp70, hsp90 and immunophilins (21;22). These conformational changes have been suggested to uncover nuclear localization signal motifs contained in the hinge region of the receptors that are necessary for recognition by the transport machinery of the cell (23). Second, members of the importin family of proteins direct the ligand-activated receptor to gated channels of the nuclear membrane which effectuate the translocation of the receptors to the nucleus. Here, the corticosteroid receptor interacts with the DNA and/or with other transcription factors to regulate gene expression by the sequential and ordered recruitment of coregulator proteins at high affinity binding sites (fig. 4). Because the transcriptional effects of the receptors are divergent and depend on the cell type, nonreceptor proteins such as transcriptional coregulators are likely to be involved in shaping their genomic actions.

Transcription is a highly controlled process of molecular interactions that requires a specific sequence of events such as initiation of transcription, elongation of RNA and termination (24;25). Ligand-activated steroid receptors, such as the GR and MR, are typically considered to modulate transcription initiation rate (26). Recently, fluorescence recovery after photobleaching (FRAP) experiments have provided insights in the kinetics of the receptors at sub-second time resolution within the nucleus (27). A proposed dynamic model is that many rapid random transcriptionally unproductive complexes are formed in conjunction to the association of the appropriate factors at specific DNA sites. These incidental random interactions have been suggested to be essential in the scanning of the genome (known as the “hit-and-run” model) (28).

2.4 Glucocorticoid response elements: recognition sites on the DNA

Both receptors recognize the same response elements in the DNA, termed glucocorticoid response element (GRE). The ‘consensus GRE’ has been empirically defined and typically is composed of two palindromic hexanucleotide half sites separated by a spacer composed of three arbitrary nucleotides (29;30). The canonical sequence is AGAACAnnnTGTTCT, but

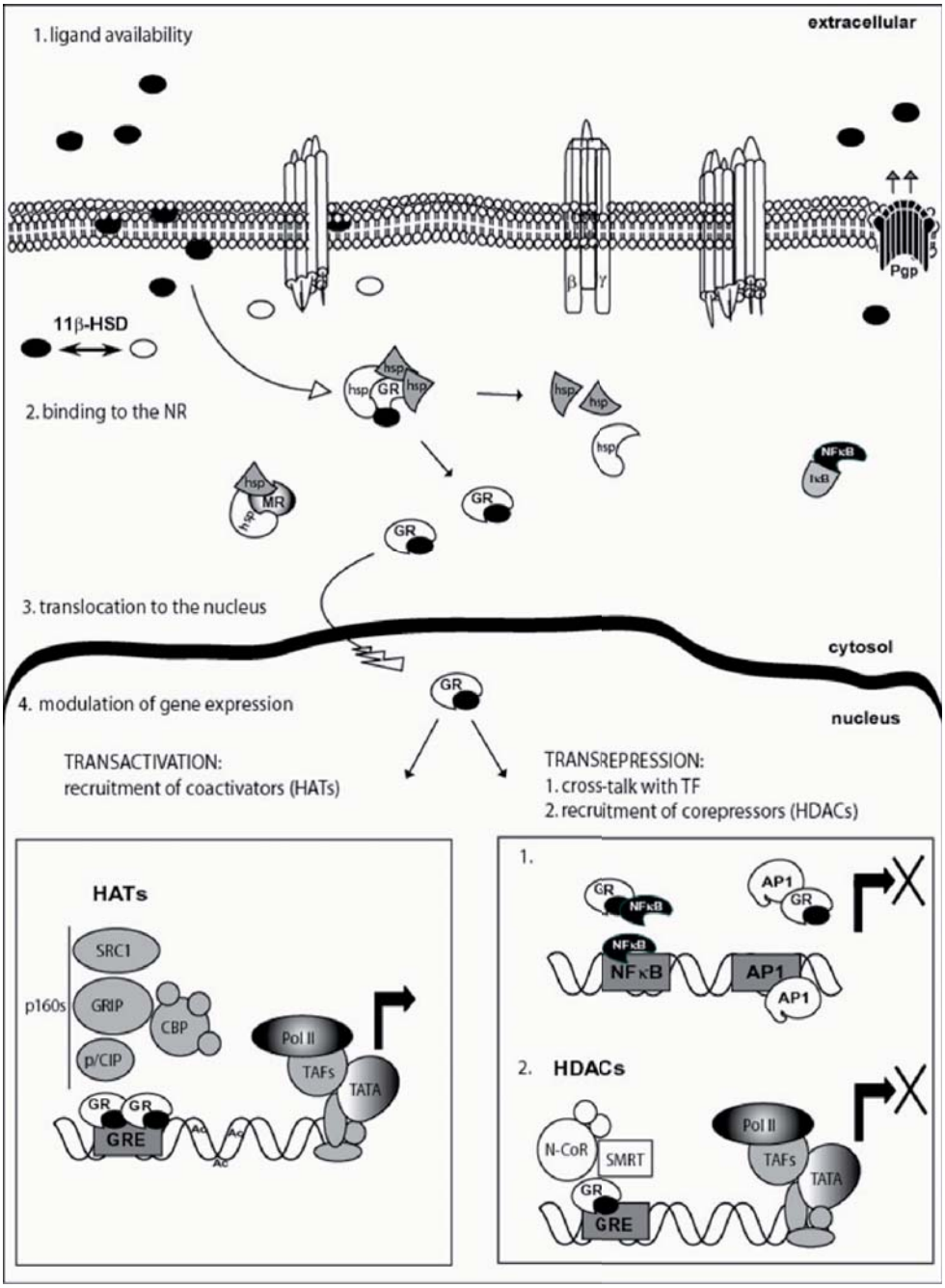

Fig. 4: Molecular mechanisms of mineralocorticoid and glucocorticoid receptor action on gene expression. First, ligand-binding induces conformational changes in the receptors which causes dissociation of chaperone proteins. Subsequently, the receptors translocate to the nucleus and either induce or repress gene expression, termed respectively transactivation or transrepression.

many variations are possible. The promoter regions of target genes may contain one or more GREs, as well as additional half sites. As a consequence of 1) cooperative binding to adjacent sites, 2) interactions with other transcription factors and 3) differences in GRE nucleotide composition, substantial differences in affinities are expected for binding of the MR and GR to different responsive genes.

Activation of the corticosteroid receptors can both result in stimulation or repression of target genes, termed transactivation and transrepression respectively. Transactivation typically involves direct binding of GR to specific DNA sites and subsequent recruitment of coregulator proteins. Transrepression is brought about by direct interference of GR with other transcriptional factors such as activator protein 1 (AP-1) and nuclear factor-κB (NF-κB). While transactivation through DNA binding requires dimerization (or multimerization) of GR, transrepression is mediated by monomers of the receptor (31). In considering transactivation by GR, it is important to note that this also occurs at composite promoters composed of several response elements not necessarily containing a GRE (32;33). The nucleotide sequence composition of the GRE and its flanking sequence are influential characteristics in determining both magnitude and nature of the response (34). Upon binding of the receptor to the GRE, allosteric changes in the receptor result in protein surfaces favoring recruitment of a selection of proteins such as coregulators.

2.4 Target genes in the HPA axis: nGREs

A particular mechanism for repression of target genes by the glucocorticoid receptor occurs via functional 'negative GREs' (nGRE). These have been identified in the promoter regions of several genes including the pro-opiomelanocortin (POMC) and corticotrophin releasing hormone (CRH) genes; the two genes produce the main peptide hormones of the HPA axis (35;36). The GR-binding region that conveys the GR-mediated repression of the cAMP-induced CRH-promoter has been identified by electrophoretic mobility shift assays (EMSA). Internal deletion of the identified nGRE and specific point mutations resulted in a loss of repression by the ligand-activated GR, indicating that DNA binding is essential for the glucocorticoid-induced repression.

The mechanism by which agonist-bound GR can mediate repression is not well-understood. The allosteric changes that proceed from binding of GR to the nGRE may favor recruitment of proteins with enzymatic activities that have adverse effects on transcription such as the corepressor proteins nuclear corepressor (N-CoR) and silencing mediator of the retinoid and thyroid hormone receptor (SMRT) (37;38). Alternatively, the location of the nGRE in the promoter is in such close proximity to a response element of a different transcription factor that sterical hindrance prevents simultaneous binding of both transcription factors. Spacing of the response elements has previously been reported to determine the nature of the response (39).This would imply competitive binding of different transcription factors at a promoter.

2.5 Neuroanatomical distribution of the corticosteroid receptors

The GR is virtually omnipresent and found at particularly high levels in the immune system, bone, lungs, liver, adipose tissue and brain (www.nursa.org/10.1621/datasets.02001) – reflecting the main clinical use and side effects of synthetic glucocorticoids. The MR is expressed in specific tissues such as brain, kidney, colon, salivary and sweat glands and is present in a large variety of cells including neurons, cardiomyocytes and adipocytes (40;41).

The effects of glucocorticoids on the brain are of particular interest, and form a challenge to

understand the basis of stress related psychopathology. GRs are present in both astrocytes and neurons, with the exception of a few areas, such as the suprachiasmatic nucleus, the site of the circadian clock. MRs are more restricted, and expressed at particularly high levels in the hippocampus, a brain region crucial for learning and memory formation. Colocalization of MR and GR is found in limbic regions such as the hippocampus. While varying expression levels of the receptor clearly affect glucocorticoid actions, their (neuro)anatomical distribution does not satisfactorily explain the cell-specific effects elicited by glucocorticoids. For example, as mentioned earlier, peripheral administration of glucocorticoids decreases CRH mRNA expression levels in the paraventricular nucleus of the hypothalamus (PVN) but concurrently increases CRH transcript levels in the central nucleus of the amygdala (fig. 2). Accordingly, adrenalectomy has the opposite effects on CRH gene expression in the CeA and PVN (42;43). The molecular mechanism by which glucocorticoids simultaneously mediate opposing effects at the same promoter in different cell types remains so far unexplained. A main objective of the work described in this thesis is to assess the role of proteins (coregulators) that may interact with corticosteroid receptors and possibly modulate the nature and the magnitude of their response.

3. Non-receptor transcriptional modulators

3.1 Nuclear receptor coregulators

Although glucocorticoids have pleiotropic effects, the target genes are to a great extent very cell type specific (44). A major determinant in imposing the effects of glucocorticoids is transcriptional coregulator protein recruitment: these proteins mediate the transduction of 'the signal' from the DNA-bound steroid receptor to the transcription machinery. Transcriptional coregulator proteins are enzymatically active proteins that reorganize chromatin environment after recruitment by the ligand-activated receptor. Regulation of gene expression by nuclear receptors requires positively and negatively acting transcriptional coregulators. Classically, coregulators have been categorized in coactivators or corepressors depending on their influence on nuclear-receptor driven transcription.

Transcriptional coregulator proteins are components of multisubunit complexes supplying the receptors with a large diversity of enzymatic activities. Protein complexes containing transcriptional coactivators provide among others histone acetyltransferase activity (HAT) which is necessary to 'unpack' the chromatin structure. On the opposite, complexes composed with corepressor proteins contain histone deacetyltransferase activity (HDAC) (37;38;45;46). In addition to their enzymatic activities, these multisubunit complexes supply specific docking surfaces for the recruitment of many different proteins among which transcriptional coregulators. In general it is thought that agonist-bound nuclear receptors have a higher affinity for protein complexes containing transcriptional coactivators, whereas antagonist-binding favors recruitment of corepressor complexes. However, recently it was found that the corepressor CNOT1 is recruited by the agonist bound nuclear receptors (47). In addition RIP140 and LCoR were also reported to induce repression in a ligand-dependent manner (48-50). All ligand-receptor complexes (agonists and antagonists) present a specific protein surface allowing interaction with transcriptional coregulator complexes and other proteins. So far ~300 nuclear receptor coregulator proteins have already been reported in literature, clearly indicating the many potential combinatorial interactions possible in the context of nuclear receptor driven transcription (51;52).

In the context of the (brain) GR and MR only few coregulators have been studied, among which the steroid receptor coactivator (SRC) proteins, and the corepressors NCoR and SMRT (53-55). Recently, a model was suggested in which coactivators and corepressors should

have opposing effects on the transcriptional activity of the GR (54). While SRC recruitment by the GR increased its transcriptional activity at a target gene, recruitment of the corepressor SMRT resulted in a loss of transcriptional activity. In parallel, additional evidence of the importance of corepressors such as N-CoR and SMRT for the brain was given by the knock-out animals.

3.2 Differential effect of SRC1 isoforms on corticosteroid receptor action

The first identified and best studied coactivator proteins are the members of the p160 steroid receptor coactivators (SRC) family (56). SRCs are considered more or less specific regulators of nuclear receptor signaling (57) (in contrast to integrator proteins such as CBP/p300), and are rate-limiting for steroid-signaling in many conditions. The family consists of three genes among which the steroid receptor coactivator 1 isoforms recently have been found to differentially affect the transcriptional activity of both corticosteroid receptors. The splice variants SRC1a and SRC1e interact with the C-terminal domain of the corticosteroid receptors with specific LxxLL motifs also known as 'nuclear receptor boxes' (NR-box) and possess two distinct activation domains which serve to enhance transcription (fig. 5A). The SRC1a and SRC1e isoforms differ only in their carboxy terminus. The most compelling differences between the two splice-variants is the additional NR box and the putative suppressor domain in the SRC1a specific sequence, which leads to differences in interactions with nuclear receptors (58). Strikingly, overexpression of SRC1a led to potentiation of the transcriptional activity of GR only at the promoter containing a single GRE and not on a promoter containing multiple GREs. On the other hand, SRC1e overexpression led to stimulation of the transcriptional activity of the GRE exclusively on a promoter containing multiple GREs, indicating the specific action of both isoforms (Fig. 5B) (57).

3.3 Neuroanatomical distribution

Since corticosteroid receptor function is critically regulated by coregulators, it is of interest to determine their expression levels in rodent brain. Recently, both SRC1 splice variants expression levels were mapped in the rat pituitary and brain (59). Both transcripts were widely detected throughout the brain. Distinct brain nuclei showed a pronounced difference in relative expression levels, suggesting differences in the modulation of the corticosteroid signaling in these areas (Fig. 5C). The most compelling differences between the two splice variants were observed in the paraventricular and ventromedial nuclei of the hypothalamus. Strikingly, the highly abundant expression of SRC1a in the PVN coincides with the site specific glucocorticoid-dependent repression of the CRH gene. Consequently, we hypothesize that the differences in coactivator expression may underlie the cell specific effects of glucocorticoids in brain. However, the expression levels and the activity of many coregulators, and their effects on corticosteroid signaling in brain remain largely unknown. This caveat forms the basis of this thesis.

4. Scope and outline of the thesis

4.1 Objective and experimental approach

A fundamental question in the neurobiology of stress is to understand how glucocorticoids can promote in discrete neural circuits processes underlying emotional and cognitive performance, while containing stress reactions elsewhere. To address this question all the experiments described in this thesis were designed to **gain insight in the molecular mechanism by which glucocorticoids mediate cell-specific effects in brain**. The main hypothesis, based on the original observation that SRC1 isoforms have distinct expression patterns in brain tissue, is that nuclear receptor coregulators contribute to the cell-specificity of

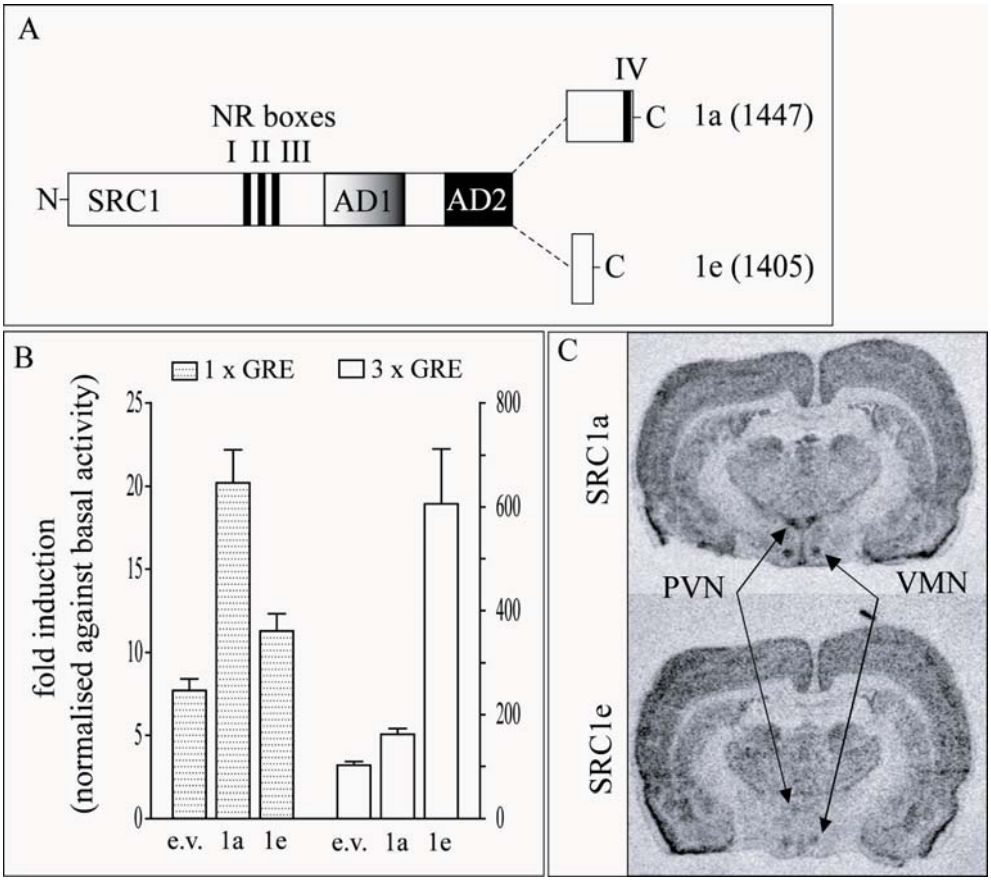

Fig. 5: (A) Schematic lay-out of the SRC1 splice variant proteins. Both isoforms interact with nuclear receptors through LxxLL motifs called nuclear receptor boxes (NR-boxes). SRC1a contains an additional NR box in its C-terminal domain. Gene expression is enhanced by the activation domains 1 and 2. Amino acid numbers of the two proteins are depicted. (B) Promoter-specific effects of steroid receptor coactivator splice variants 1a and 1e on the transcriptional activity of the glucocorticoid receptor. (C) in situ hybridisation of the SRC1 splice variants in rodent brain. Distinct brain regions have profound differences in expression levels of both splice variants. PVN: paraventricular nucleus of the hypothalamus; VMN: ventromedial nucleus of the hypothalamus.

glucocorticoids. Recent studies on the role of coregulators in steroid-driven transcription provided evidence of the importance of these proteins *in vitro*. These studies led to a central postulate stating that corepressor and coactivator proteins determine the dose response curve of agonist-bound steroid receptors (fig. 6) (53). Consequently, corticosteroid signaling depends on the actual expression of MR and/or GR and coexpression with coregulators. The experiments described in this thesis were designed to address three specific questions:

(1) Are corepressors differentially expressed in the rodent brain? It was recently shown that coactivators are expressed in the rodent brain but what about corepressors? Expression of corepressors was addressed by mapping the distribution of the two best-described corepressors at the mRNA and protein level in the rodent brain. This was assessed by means of *in situ* hybridization and dual-immunofluorescence histochemistry on thin rat brain sections.

(2) What is the effect of coactivator or corepressor overexpression on GR-mediated transcription regulation? To gain insight in the function of coregulator proteins in the transcriptional activity of the GR at an endogenous promoter, *i.e.* the human CRH promoter, the coregulators were individually overexpressed in cultured cells (AtT-20 cells: mouse anterior pituitary cells that endogenously express GR). In this system, GR-mediated control of CRH expression was assessed in at varying cellular concentrations of coregulators. Regulation of the CRH-promoter was studied because it is an essential glucocorticoid target gene critically involved in the regulation of the HPA axis activity.

(3) How can we study coregulator recruitment *in vivo?* In order to address coregulator recruitment of GR *in vivo*, a recently described ingenious experimental approach termed 'chromatin immunoprecipitation' assay was set up. Subsequently, using this technique the proximal promoter of the rat glucocorticoid-induced leucine zipper (GILZ) gene was scanned for glucocorticoid response elements (GREs). Additionally, regulation by glucocorticoids of the GILZ gene in rat brain was tested by *in situ* hybridization.

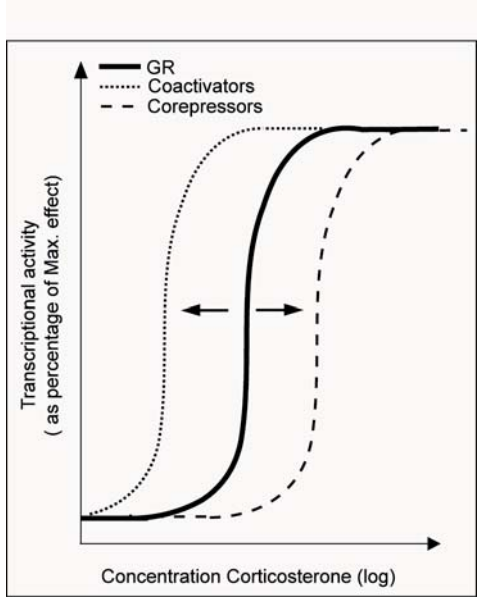

Fig 6: Model for control of dose-response curve of agonist bound corticosteroid receptor. Corepressor recruitment induces a left-shift of the dose-response curve whereas coactivators have opposing effects. The model is based on previously described work by Szapary et al. 1999.

4.2 Outline of the thesis

In Chapter 2, we describe the neuroanatomical distribution of two functionally distinct corepressors involved in the regulation of gene expression by steroid receptors. Furthermore, we provide evidence for colocalisation of N-CoR and SMRT proteins within the nucleus of glucocorticoid target cells in distinct brain nuclei critically involved in the regulation of the HPA axis, among which the paraventricular nucleus of the hypothalamus (PVN).

In Chapter 3, based on the uneven distribution of a number of coregulators in CRH expressing cells previously observed, we tested the hypothesis that these proteins are involved as mediators in the glucocorticoid induced repression of the CRH promoter. Several coregulators previously identified to be expressed in the rodent brain, *i.e.* SRC1a, SRC1e, N-CoR and

SMRT, were individually tested in a well-established model of GR-mediated repression.

In Chapter 4 the cross talk between the two main signalling pathways involved in activation and repression of CRH mRNA expression: cyclic AMP (cAMP) and GR is studied. Activation of the GR shortly after cAMP-induction of the CRH gene is essential for effective repression. This may be relevant since the time between activation of the two signaling cascades *in vivo* may largely vary in the context of a stressful situation.

To further characterize the role of coregulators in brain, we describe in Chapter 5 a method that permits identification of GR-binding regions in the promoter region of target gene. In addition, we explored the possibilities of using the glucocorticoid-induced leucine zipper (GILZ) gene as a candidate for chromatin immunoprecipitation (ChIP) assays on brain tissue to address issues such as coregulator recruitment *in vivo*.

Finally in Chapter 6 a synopsis of all major findings is given. In extension, the data presented in this thesis are discussed in the context of the 'biology of stress' and the potential implications for safer drug design are presented.

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