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

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Voor Selwyn & Jonas

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Abbreviations

CeA	Central nucleus of the amygdala
CORT	Corticosterone
CRE	cAMP response element
CREB	CRE-binding protein
CRH	Corticotrophin-releasing hormone
DEX	Dexamethasone
GC	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA axis	Hypothalamus pituitary adrenal axis
LC	Locus Coeruleus
N-CoR	Nuclear corepressor
nGRE	negative Glucocorticoid response element
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
SMRT	Silencing mediator of the retinoid and thyroid hormone receptor
SRC	Steroid receptor coactivator

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.

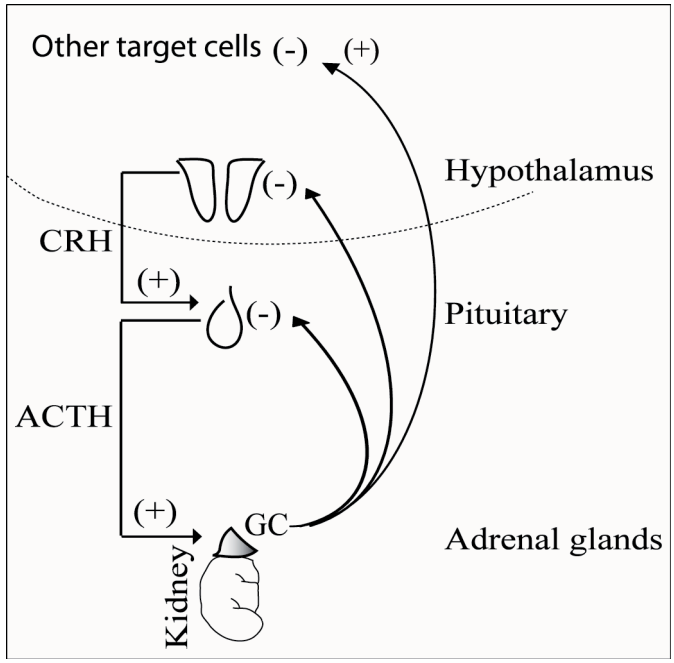

Fig.1: Representative scheme of the hypothalamus-pituitary-adrenal axis (HPA axis). Upon activation, parvocellular cells in the PVN release corticotrophin releasing hormone (CRH). CRH stimulates the expression of adrenocorticotropin hormone (ACTH) which in turn enhances glucocorticoid (GC) secretion 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).

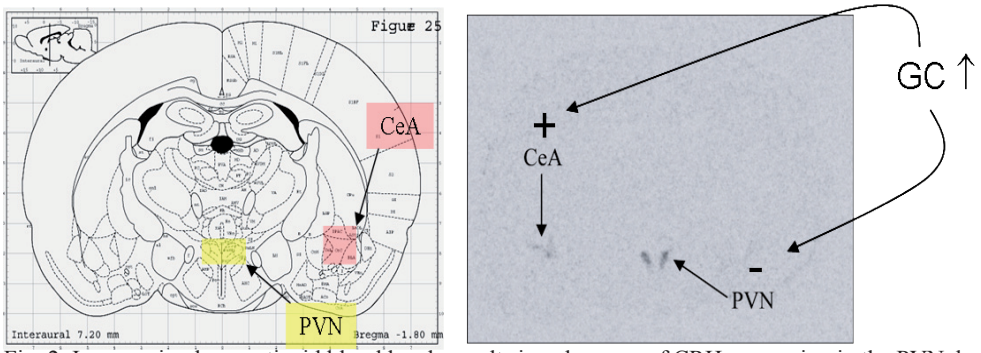

Fig. 2: Increase in glucocorticoid blood levels results in a decrease of CRH expression in the PVN, but concurrently stimulates CRH expression in the CeA in the rodent brain. The mechanism(s) by which glucocorticoids can exert cell-specific opposing effects on CRH gene expression in the rodent brain 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.

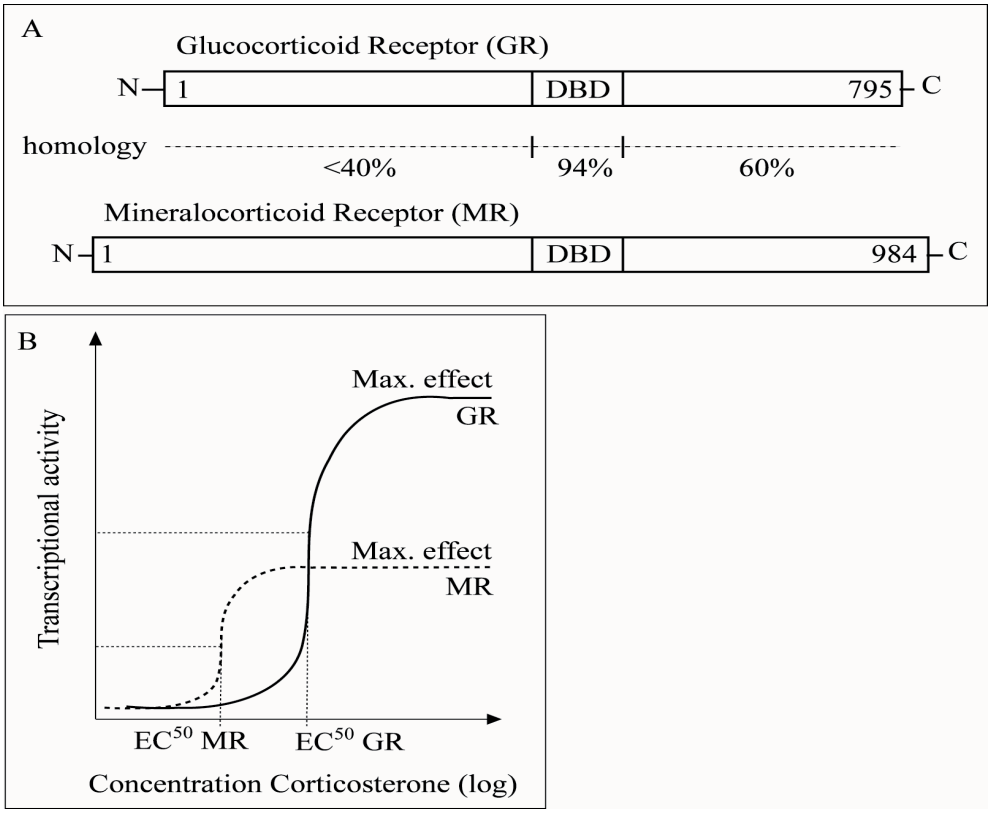

Fig. 3: (A) Schematic lay-out of the glucocorticoid and mineralocorticoid receptors (GR and MR) and homology in their amino acid sequence. Both receptors belong to the large superfamily of nuclear receptors and contain three distinctive domains: the N-terminal, the DNA-binding and C-terminal domains. (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

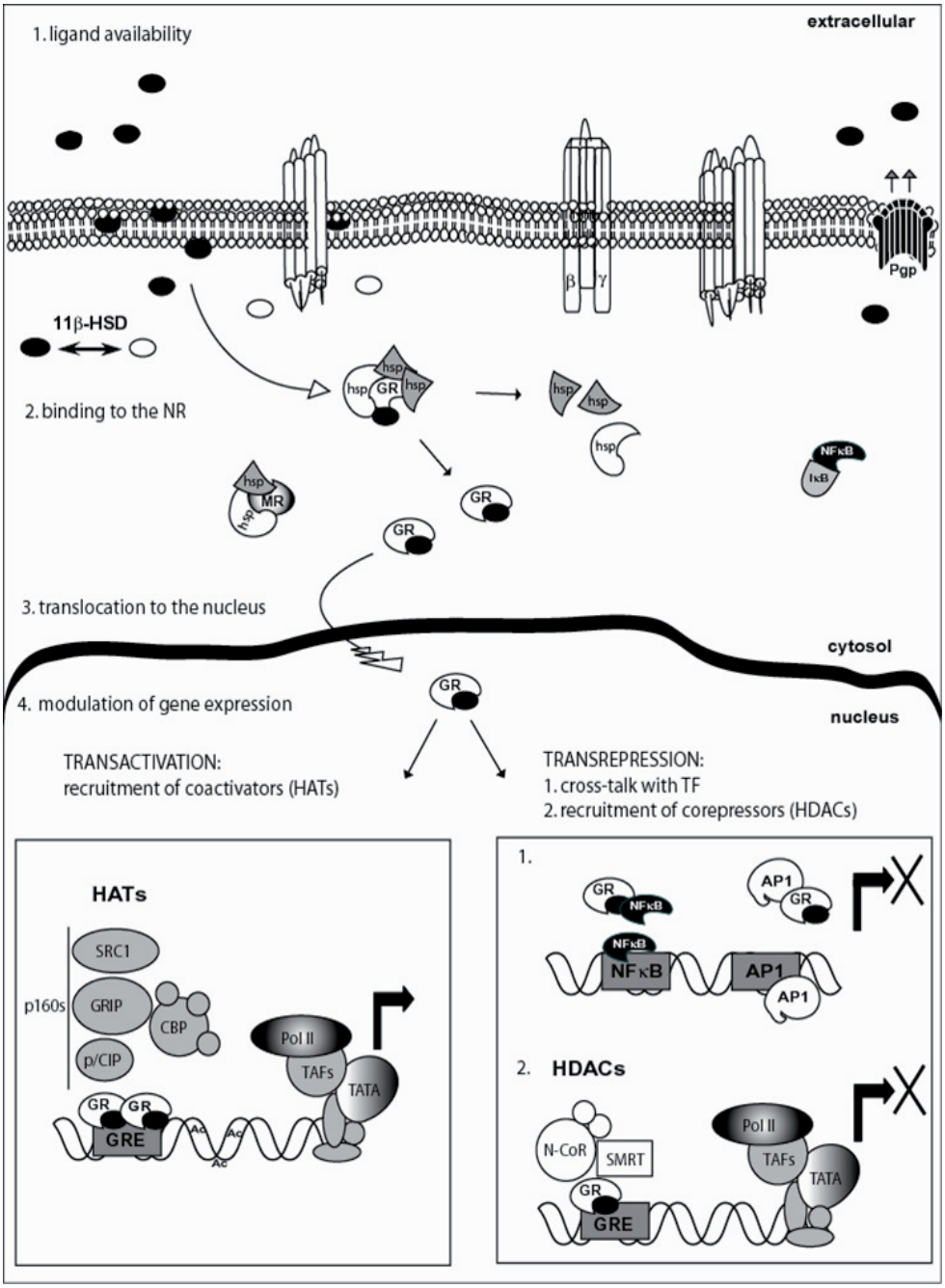

Fig. 4: Molecular mechanisms of mineralocorticoid and glucocorticoid receptor action on gene expression. First, ligand-binding induces conformational changes in the receptors which causes dissociation of chaperone proteins. Subsequently, the receptors translocate to the nucleus and either induce or repress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

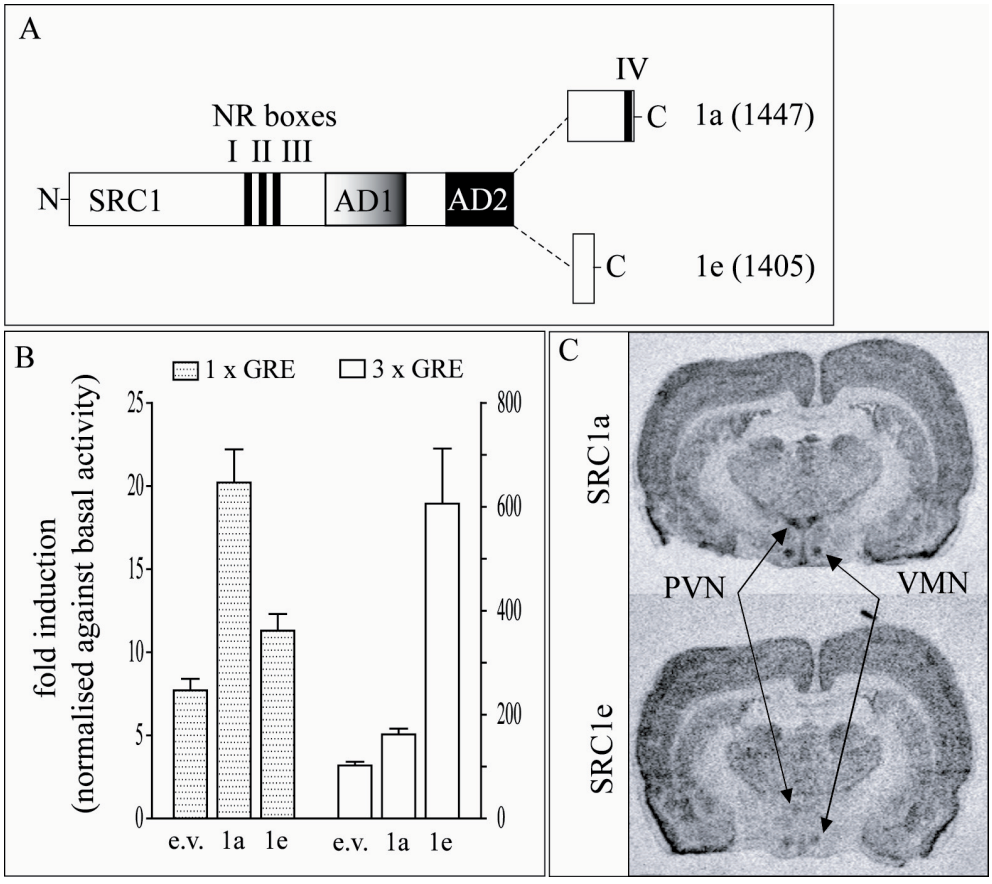

Fig. 5: (A) Schematic lay-out of the SRC1 splice variant proteins. Both isoforms interact with nuclear receptors through LxxLL motifs called nuclear receptor boxes (NR-boxes). SRC1a contains an additional NR box in its C-terminal domain. Gene expression is enhanced by the activation domains 1 and 2. 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.

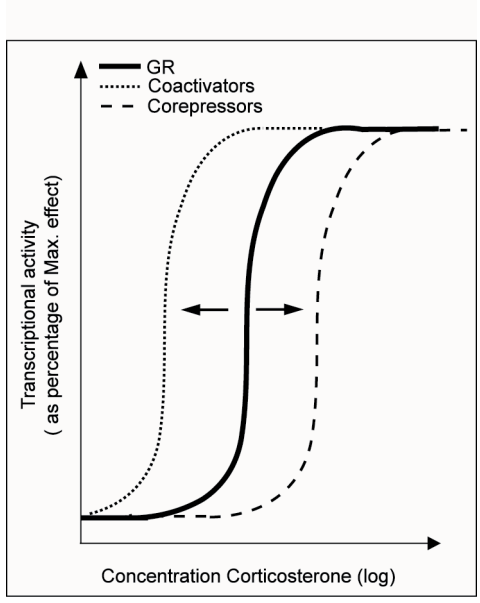

Fig 6: Model for control of dose-response curve of agonist bound corticosteroid receptor. Corepressor recruitment induces a left-shift of the dose-response curve whereas coactivators have opposing effects. The model is based on previously described work by Szapary et al. 1999.

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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Chapter II

Neuroanatomical distribution and colocalisation of nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic and thyroid (SMRT) receptors in rat brain

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Abstract

The two structurally related Nuclear Receptor Corepressor (N-CoR) and Silencing Mediator of Retinoid and Thyroid receptors (SMRT) proteins have been found to differentially affect the transcriptional activity of numerous nuclear receptors, such as thyroid hormone, retinoic acid and steroid receptors. Because of the numerous effects mediated by nuclear receptors in brain, it is of interest to extend these *in vitro* data and to explore the cellular distribution of both corepressors in brain tissue. We therefore examined, using *in situ* hybridisation, whether the relative abundance of these two functionally distinct corepressors differed in rat brain and pituitary. We find that although both N-CoR and SMRT transcripts are ubiquitously expressed in brain, striking differences in their respective levels of expression could be observed in discrete areas of brain stem, thalamus, hypothalamus and hippocampus. Using dual-label immunofluorescence, we examined in selected glucocorticoid sensitive areas involved in the regulation of the hypothalamus-pituitary-adrenal axis activity, the respective protein abundance of N-CoR and SMRT. Protein abundance was largely concurrent with the mRNA expression levels, with SMRT relatively more abundant in hypothalamus and brain stem areas. Colocalisation of N-CoR and SMRT was demonstrated by confocal microscopy in most areas studied. Taken together, these findings are consistent with the idea that the uneven neuroanatomical distribution of N-CoR and SMRT protein may contribute to the site-specific effects exerted by hormones, such as glucocorticoids, in the brain.

1. Introduction

Nuclear receptors are ligand-inducible transcription factors that modulate gene expression by specifically binding to responsive elements in promoter regions of target genes. The nuclear receptor family consists of type I receptors (e.g. estrogen, progesterone, androgen, mineralocorticoid and glucocorticoid receptors (ER, PR, AR, MR and GR)), type II receptors (e.g. retinoic acid, thyroid hormone and vitamin D receptors (RAR, TR and VDR)) and orphan receptors (1). These receptors are widely distributed in an uneven manner over many brain regions. The neuroanatomical distribution of the receptors does not satisfactorily explain site-specific effects elicited by their cognate ligand. Previously we have reported an uneven distribution for the two splice variants of the most abundantly expressed p160 coactivator in the rodent brain that have distinct effects on for example the GR and MR (2,3).

Two structurally related but functionally distinct proteins, Silencing Mediator of the Retinoid and Thyroid receptor (SMRT) and Nuclear Corepressors (N-CoR) have both emerged as key players in the mechanism of nuclear receptor mediated gene repression (4,5). N-CoR and SMRT repress gene expression by binding directly to the nuclear receptor and facilitating the recruitment of chromatin remodelling enzymes. They are well-documented, structurally-related corepressor proteins of approximately 270 kDa, which repress gene transcription by recruiting histone deacetylases to the proximity of the nuclear receptor and forming corepressor complexes (6).

While both corepressors originally were defined as repressors of type II nuclear receptors, such as thyroid hormone receptor (TR) and retinoic acid receptor (RAR), they have now been shown to alter the transcriptional activity of steroid receptors *in vitro* (7-9). For example, the dose-response curve of agonist-activated GR and MR is shifted to the right in presence of corepressor proteins. This was observed for endogenous as well as for synthetic ligands (10). Consequently, it has been proposed that at sub-saturating levels of steroids, the presence of corepressor proteins will influence the genomic effects of the ligand-activated nuclear receptors. Although N-CoR and SMRT share structural similarities, it is noteworthy that they have been shown to differentially affect nuclear receptor signalling (8). Therefore, we hypothesise that their relative abundance might determine the effects of steroids on gene transcription. Because of the numerous effects mediated by nuclear receptors in brain, it is of interest to extend these *in vitro* data and to explore the cellular distribution of both corepressors in brain tissues.

We mapped the expression and the relative abundance of N-CoR and SMRT mRNA in rat brain and pituitary gland. Our particular interests are the site-specific effects elicited by glucocorticoid hormones in the brain (for review (11)) and the regulation of the hypothalamus-pituitary-adrenal axis (HPA-axis). Therefore, we demonstrated the respective levels of expression on protein level by means of immunofluorescence in GR and/or MR expressing areas that are important in the regulation of the HPA-axis activity.

2 Material & Methods

2.1 Animals and tissue preparation

Adult male Wistar rats (240g; n=8) were obtained from Charles River Laboratories (Germany). All animals were group-housed (n= 4 per cage) had *ad libitum* access to food and water, and were maintained under controlled conditions, on a 12:12 hour light cycle (lights on from 08.00 to 20.00 h). One week after arrival, all rats were sacrificed in the morning (between 9:00 and 11:00 a.m.). The brains were snap frozen in isopentane (cooled in an ethanol-dry ice

bath) and the pituitaries were frozen on dry ice. All tissues were stored at -80°C until further use. Experiments were carried out with the approval of the Animal Care Committee of the Faculty of Medicine, Leiden University, The Netherlands (DEC nr. 03130).

2.2 In situ hybridisation

Brains of male Wistar rats were used for the *in situ* hybridization procedure (n=4). Thin sections of both brains (20µm) and pituitaries (10 µm) were cut on a cryostat (Leica CM3050S), thaw-mounted on poly-L-lysine (Sigma) coated slices, and stored at -80°C. The sections were fixed for 30 min. in freshly made 4% para-formaldehyde (Sigma) in phosphate buffered saline (PBS, pH 7.4), rinsed twice in PBS, acetylated in triethanolamine (0.1 M, pH 8.0) with 0.25% acetic anhydride for 10 min, rinsed for 10 min in 2 x SSC (SSC: 150 mM sodium chloride, 15 mM sodium citrate), dehydrated in an ethanol series, air dried and stored at room temperature until the *in situ* hybridisation. N-CoR and SMRT riboprobes were amplified by PCR on genomic DNA. For N-CoR, a segment of 482 nucleotides (Genbank Accession number U35312; nucleotide 4715 to 4654) and for SMRT, a segment of 381 nucleotides (Genbank Accession number AF113001; nucleotide 7217 to 7597), was inserted in a PGEMT-easy vector. These murine fragments contained minimal cross-homology and showed 96% identity with corresponding rat mRNA. Hybridisation mix consisted of 50% formamide, 20% dextran sulfate, 1.2 mM EDTA (pH 8.0), 25 mM sodium phosphate (pH 7.0), 350 mM sodium chloride, 100 mM DTT and, 1% Denhardt's, 2% RNA-DNA mix, 0.2% nathiosulfate and 0.2% sodium dodecyl sulfate. A 100 µl aliquot of hybridisation mix containing 2.5 x 10^6 dpm of N-CoR or SMRT riboprobe was added to each section. Coverslips were brought on the slides which were hybridised overnight in a moist chamber at 55°C. The next morning, coverslips were removed and the sections washed in graded salt/formamide at optimised temperature. After the washing steps, sections were dehydrated in a series of ethanol baths and air dried.

The N-CoR and SMRT hybridised sections were apposed to Kodak BioMax MR film for 13 and 7 days, respectively. After development of the films the sections were counter-stained with 0.5% cresyl violet for anatomical analysis. Control sections were treated identically to experimental sections except that sense riboprobes were used. Control sections did not give signal above background.

2.3 Immunofluorescence and confocal laser scanning microscopy

Brains of adult male Wistar rats were used for the immunohistochemistry experiments (n=4). Thin brain sections (20µm) were cut on a cryostat (Leica CM3050S), thaw-mounted on poly-L-lysine (Sigma) coated slices, and stored at -80°C until further use. Slides were allowed to thaw at 4°C during 20 min. prior to fixation. The sections were fixed during 10 min. in pre-chilled methanol/acetone/water [40:40:20 (v/v/v)] solution at 4°C. After fixation, sections were washed three times in 1x phosphate-buffer saline with 0.2% Tween (1 x PBST pH 7.4) and blocked 60 min in 5% normal donkey serum (NDS) at room temperature. Incubations with anti-N-CoR (Santa Cruz biotechnologies; goat polyclonal C-20, 1:50) and anti-SMRT (Upstate; rabbit polycolonal cat. # 06-891, 1:200) primary antibodies were performed overnight at 4°C. The next morning, the sections were allowed to acclimatise for one hour to room temperature, washed three times in 1 x PBST. Detection of N-CoR and SMRT positive cells was realized with FITC conjugated donkey-anti-goat IgG (Santa Cruz biotechnologies; sc-2024, 1:50) and Cy3 conjugated donkey-anti-rabbit IgG (Upstate; 1:150), respectively. Sections were incubated with the secondary antibodies for 60 min. at 37°C. After incubation, sections were washed in 1 x PBST and counter-stained for 10 min. with Hoechst 33528, and washed four times (5 min.) in 1 x PBST. All sections were embedded in polyaquamount

(Polysciences, inc.) and observed with an immunofluorescence microscope (Leica DM6000) or a confocal laser scanning microscope (Bio-Rad Radiance 2100MP). Control sections were incubated with equal amounts of non-immune rabbit and goat sera, which were used as substitute for the primary antibodies. Nuclear immunoreactivity was used for quantification. The amount of non-specific nuclear immunoreactivity was found to vary for each brain area studied and consequently was deducted from the total signal obtained within the corresponding area. For confocal microscopy, Bio-Rad Radiance apparatus equipped with a HeNe and Argon laser was used and the image analysis was performed with a Kalman collection filter (2 scans).

2.4 Analysis and Quantification

For analysis of the relative optical density (ROD) for *in situ* hybridisation and the immunofluorescence, ImageJ 1.32j software (NIH, USA) was used. The mRNA expression was measured for four rats, guided by cresyl-violet counterstained sections and a rat brain atlas (12). The autoradiographs were scanned (1000 dpi) and saved as uncompressed 8-bit grey-scale tiff files. Images of adjacent sections hybridised with N-CoR and SMRT riboprobes were opened in ImageJ to allow visual comparison during analysis. Signal was obtained by subtracting the sense signal from the anti-sense signal. To allow brain wide comparison of expression levels for each transcript, signals were normalized for each rat against the darkest signal (the granular cell layer of the dentate gyrus (DG) in both cases) as previously described (2). The average expression level per area was then assigned to a category according to the percentile of their grey values: +/- < 25%; + 25-35 %; ++ 35-60 %, +++ 60-90 %; ++++ > 90%. Measures for relative abundance of the two transcripts was obtained by dividing the mean relative optical densities (ROD) measured for N-CoR by the mean ROD measured for SMRT for the selected glucocorticoid sensitive areas.

For the evaluation of the immunofluorescent signal a more limited number of brain regions were evaluated for practical reasons. Images were captured and saved as uncompressed tiff files. Per brain region, the collected images for N-CoR (green), SMRT (red) and the Hoechst staining (blue) were merged. Guided by the Hoechst-stain, nuclear optical density was measured. Immunoreactivity of multiple individual cells was measured per brain region. Non-specific signal (non-immune sera) was measured for each region and subtracted from the total signal to obtain the specific signal. An equal amount of cell nuclei were measured per region per rat. Differences between brain areas in corepressor stoichiometry were determined by taking the ratio of N-CoR and SMRT immunoreactive signal for specific glucocorticoid sensitive areas.

2.5 Statistics

One-tailed non-parametric Wilcoxon Signed Rank Test was used to statistically assess differences in the relative protein abundance, as it could be predicted from the mRNA mapping. Differences were considered significant at $p < 0.05$.

3. Results

3.1 N-CoR and SMRT mRNA expression in brain

N-CoR and SMRT transcripts were ubiquitously detected in brain tissue. Representative SMRT hybridisation autoradiographs (fig.1) and semi-quantification of the signal intensity are summarised in table 1.

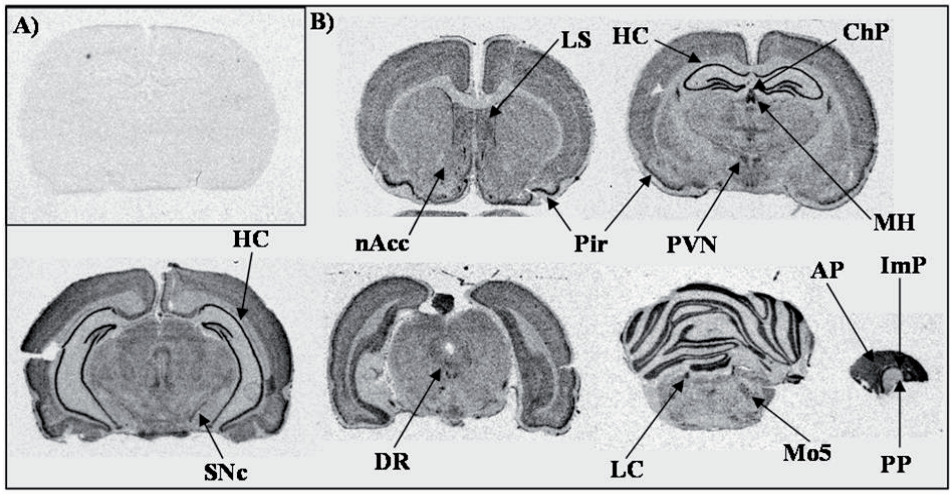

Fig.1 Specificity of hybridisation with 35S labelled sense riboprobe (A). Expression of SMRT mRNA in a series of coronal sections of the rat brain (B). LS, lateral septum; HC, hippocampus; ChP, choroid plexus; Pir, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; MH, medial habenula; SNc, substantia nigra, compact part; DR, dorsal raphe; LC, locus coeruleus; Mo5, motor trigeminal nucleus; AP, anterior pituitary; PP, posterior pituitary; ImP, intermediate lobe; nAcc, nucleus accumbens core.

Although we found overlapping pattern of distribution for both N-CoR and SMRT, clear differences in their relative transcript abundance were observed in several brain regions (fig.2). The anterior pituitary showed a homogenously distributed signal of both SMRT and N-CoR (fig.1).N-CoR hybridisation signal was high in the hippocampus (60-90%) with the granular cell layer of the DG exhibiting the strongest signal (set at 100%). The overall N-CoR anti-sense hybridisation level was markedly higher in the thalamus (35-60%) than in the hypothalamus (25-35%). Cortical areas and the cerebellar lobules showed high, homogenously distributed, hybridisation signals (60-90%). Brain stem nuclei signals, such as for the locus coeruleus and the oculomotor nucleus, hardly exceeded background levels (<25%) (fig.2).

SMRT hybridisation signal was very high in all hippocampal subregions (CA1-CA3 >90%). Cortical areas and cerebellar lobules exhibited homogenous moderate (35-60%) and high SMRT hybridisation degrees (60-90%), respectively. Hypothalamic nuclei, such as the suprachiasmatic and the supraoptic nucleus of the hypothalamus, clearly showed very high levels of SMRT hybridisation (>90%). Most motor nuclei studied, among which the substantia nigra compact part (SNc), the oculomotor and facial nucleus, showed high levels of SMRT hybridisation (60-90%). The hybridisation signal for SMRT in the thalamus was low to moderate (25-60%). Brain stem nuclei such as the locus coeruleus (LC), exhibited high signal (60-90%). As *in situ* hybridisation is a semi-quantitative technique, N-CoR and SMRT levels of expression can not be compared in one brain area.

	N-CoR	SMRT		N-CoR	SMRT
CORTICAL AREAS			HYPOTHALAMUS		
Frontal cortex, area 2	+++	++	Suprachiasmatic nucleus	++	++++
Tenia tecta	++	++	Supraoptic nucleus	++	++++
Olfactory tubercle	+++	+++	Paraventricular nucleus of the hypothalamus		
Piriform cortex	+++	++++	magnocellular part	+	+++
			parvocellular part	+	+++
HIPPOCAMPUS			Ventromedial hypothalamic nucleus	++	+++
Dentate gyrus = 100%			Arcuate hypothalamic nucleus	+++	+++
Hippocampus CA3 – pyramidal layer	+++	++++	Anterior hypothalamic area	+	++
Hippocampus CA2 – pyramidal layer	+++	++++	Posterior hypothalamic nucleus	++	++
Hippocampus CA1 – pyramidal layer	+++	++++	Supramammillary nucleus	++	+++
			Medial supramammillary nucleus	++	+++
AMYGDALA COMPLEX					
Dorsal endopiriform nucleus	++	++	MOTOR		
central amygdaloid nucleus	++	++	Oculomotor nucleus	+	+++
Lateral amygdaloid nucleus	++	++	Facial nucleus	+/-	+++
			Substantia nigra, reticular part	+/-	+
SEPTAL COMPLEX			Substantia nigra, compact part	++	+++
Septohippocampal nucleus	++	+++	Motor trigmenial nucleus	+	+++
Lateral septal nucleus, ventral	+	++	RETICULAR CORE		
Lateral septal nucleus, dorsal	+	++	Dorsal raphe nucleus	++	++
Bed nucleus of the stria terminalis,	++	++	Locus coeruleus	+/-	+++
medial division, posterointermediate part			Pontine nuclei	+	++
			Pontine reticular nucleus, oral part	+/-	+
BASAL GANGLIA					
Caudate putamen	++	++	BRAINSTEM SENSORY		
Accumbens nucleus, core	++	++	Mesencephalic trigeminal nucleus	+	+++
Globus pallidus	+/-	+			
			PRE-& POSTCEREBELLAR NUCLEI		
THALAMUS			Red nucleus	+	+++
Paraventricular thalamic nucleus	+++	+++			
Paratenial thalamic nucleus	++	++	Choroid plexus	++	++
Reticular thalamic nucleus	++	++	Cerebellar lobule 2	+++	+++
Zona incerta	++	++			
Medial habenular nucleus	+++	+++	PITUITARY		
Ventrolateral thalamic nucleus	++	+	Anterior pituitary	+++	++++
Ventral lateral geniculate nucleus,			Intermediate lobe	+++	++++
magnocellular part	++	+	Posterior pituitary	+/-	+/-

tabel 1. Regional expression of N-CoR and SMRT mRNA in the rat brain and pituitary. Prior to the calculation of the mean (n= 4), the signal was normalised per rat against the highest in situ hybridisation signal, i.e. the signal of the granular cell layer of the DG in all cases. Signal in the DG was set at 100% (scale is % of DG signal: +/- < 25%; + 25-35 %; ++ 35-60 %, +++ 60-90 %; ++++ > 90%).

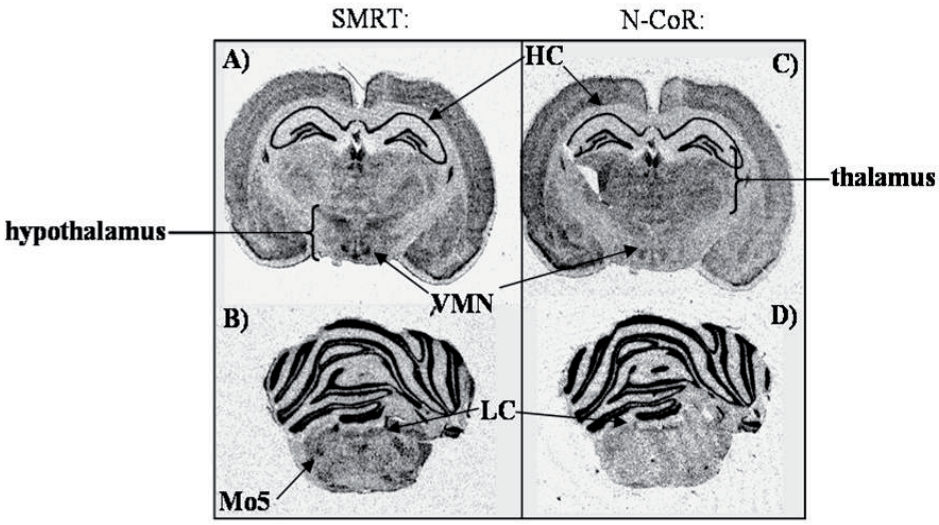

Fig. 2 Adjacent sections of SMRT (A and B) and N-CoR (C and D). SMRT and N-CoR show substantial differences in their respective levels of mRNA abundance in certain brain areas. The most striking differences are high levels of SMRT expression in hypothalamus and brain stem and high N-CoR expression in thalamus. HC, hippocampus; VMN, ventromedial nucleus; LC, locus coeruleus; Mo5, motor trigeminal nucleus.

In line with our working hypothesis that the modulation of gene transcription by glucocorticoids is dependent on the type and amount of corepressor present, we calculated the relative abundance of the corepressors by dividing the mean relative optical densities measured for N-CoR by the mean relative optical densities measured for SMRT in a number of selected glucocorticoid sensitive areas, *i.e.* hippocampal subfields, the hypothalamic paraventricular nucleus (PVN), the serotonergic dorsal raphe nucleus (DR), and the noradrenergic locus coeruleus (table 2).

Brain area	N-CoR/SMRT
CA3	0.63 ± 0.05
DG	**0.77 ± 0.06**
PVNp	0.64 ± 0.01
DR	0.61 ± 0.06
LC	**0.33 ± 0.04**

Table 2. Relative transcript abundance (± sem). The ROD of the signal obtained for N-CoR was divided by the ROD obtained for SMRT in 5 selected glucocorticoid sensitive areas. The LC has the lowest ratio of the areas studied indicating that it is a SMRT-enriched brain region.

This has two major advantages: 1) it corrects for the inherent differences in signal intensity observed between brain nuclei due to varying cell densities and 2) generates an adequate value for comparison of the relative abundance of both transcripts in different areas. The data show that the dentate gyrus is, relatively to the other areas studied, enriched in N-CoR mRNA, while the LC is SMRT-enriched; reflecting the overall hardly detectable N-CoR signals in brain stem nuclei (fig. 2).

3.2 N-CoR and SMRT protein expression in brain

The distribution of cells double-labeled for N-CoR and SMRT was in all areas studied in line with their respective levels found on mRNA. Representative pictures are shown in fig 3. Interestingly, specific cytoplasmic staining for N-CoR, but not SMRT, was observed in pyramidal cells in cortical areas and hippocampus (fig. 3b). This signal was observed but not quantified. Measurement of the nuclear immunoreactivity signal is summarised for N-CoR and SMRT in fig. 4a and fig. 4b, respectively.

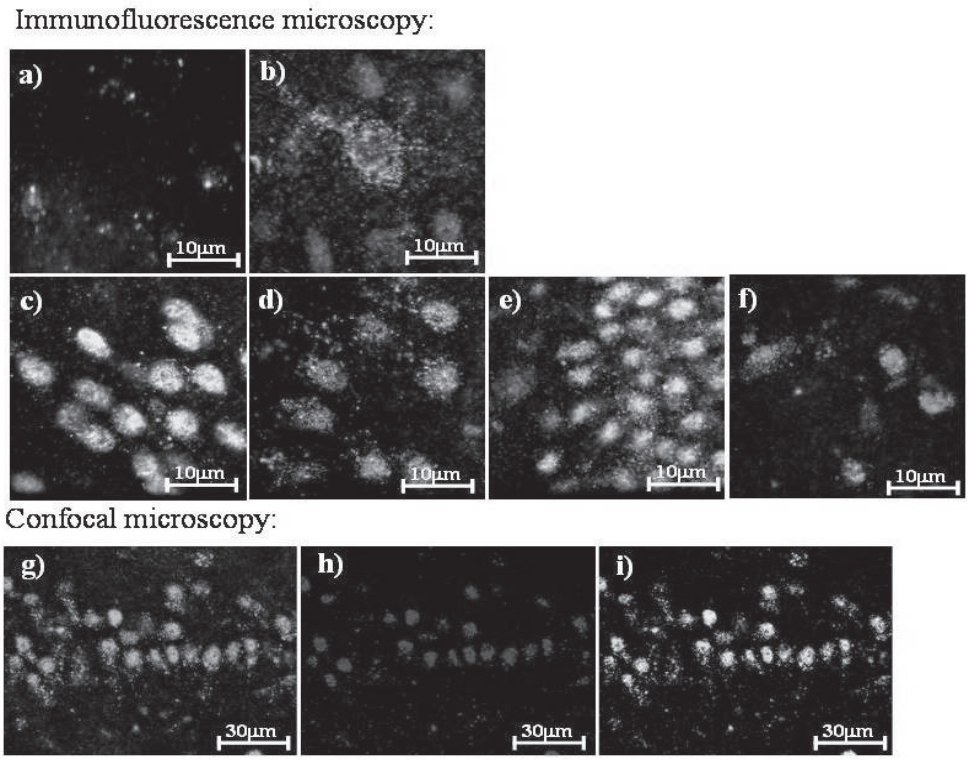

Fig. 3 Dual-immunofluorescence images for N-CoR and SMRT. The merged images show N-CoR (FITC:green) and SMRT (Cy3:red). A: Control IgG image of the aspecific immunoreactivity of non-immune sera. B: Cytoplasmic expression of N-CoR is observed in the pyramidal neurons of the frontal cortex. C-F: Relative expression of N-CoR and SMRT differs in the piriform cortex (c), CA3 (d), dentate gyrus (e) and the locus coeruleus (f). G-I: Confocal microscopy image of N-CoR (g) and SMRT (h) in the nucleus of the CA1 neurons. Colocalisation of the corepressors is shown in yellow (i). (see colour image page 123).

In general the distribution of N-CoR immunoreactivity (N-CoR-ir) paralleled that of its mRNA expression. N-CoR protein was predominantly detected in regions of the forebrain while N-CoR-ir hardly exceeded background levels in brain stem nuclei such as the LC (fig. 4a). In the hypothalamus, N-CoR-ir was higher then expected based on the signal measured by *in situ* hybridisation (fig. 2c). N-CoR-ir was only moderate in all hippocampal subregions, but high in another cell-dense area, the piriform cortex (Pir). Nevertheless, the relative N-CoR-ir within the subregions of the hippocampus paralleled the mRNA expression with the cells of the DG showing a higher N-CoR-ir than the CA3 (table 1). N-CoR-ir was similar in the cells of the DR and the parvocellular part of the paraventricular nucleus (PVNp).

SMRT protein distribution also matched the expression pattern observed for *in situ* hybridisation. SMRT immunoreactivity (SMRT-ir) was clearly discernible in hypothalamus, brain stem, and to a lesser extent, in the subregions of the hippocampus (fig 4b). In a similar manner as for N-CoR-ir, SMRT-ir was high in the Pir and moderate in the subregions of the hippocampus. Again, SMRT-ir within the subregions of the hippocampus matched the mRNA distribution; with the cells of the CA3 exhibiting the highest immunoreactivity. The highest SMRT-ir was observed in the magnocellular part of the paraventricular nucleus (PVNm). SMRT-ir in the PVNp and the DR was found to be comparable indicating that both regions do not significantly differ in their SMRT content. In contrast, SMRT-ir was approximately 1.5 times higher in the LC than in the DR.

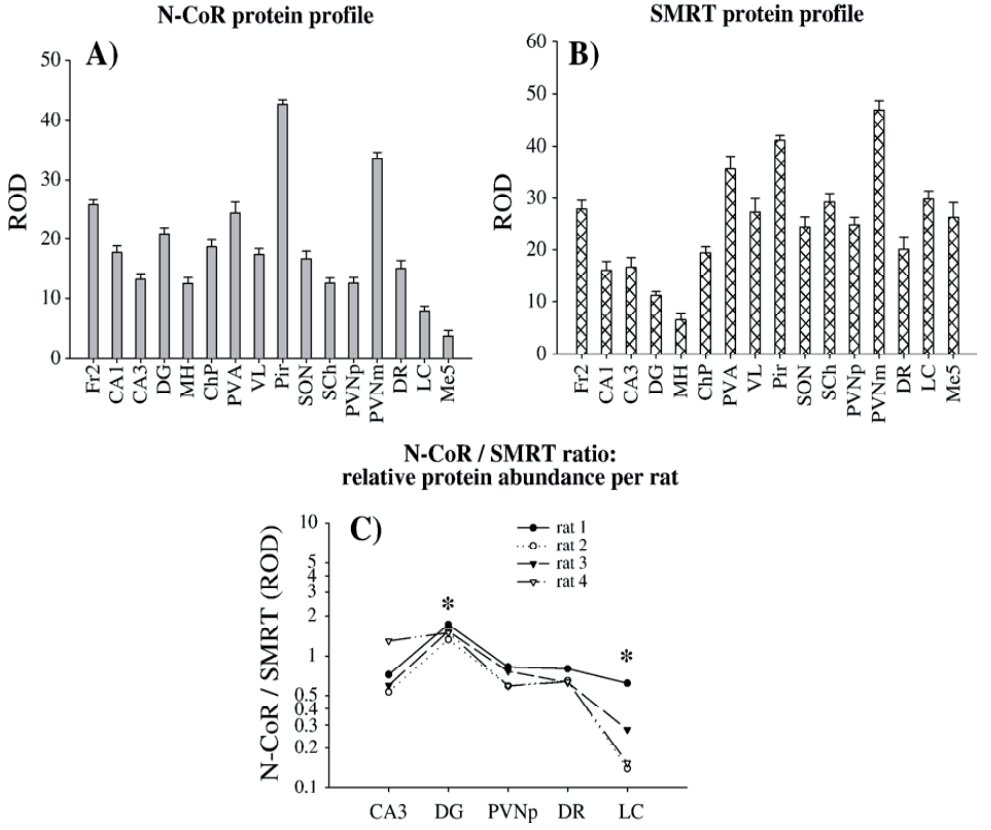

Fig. 4 N-CoR and SMRT protein expression in different areas. A,B: N-CoR (A) and SMRT (B) immunoreactivity as mean relative optical density (ROD) and standard error of the mean. Fr2, frontal cortex area 2; CA1 and CA3, hippocampus CA1 and CA3-pyramidal layer; DG, dentate gyrus; MH, medial habenula; ChP, choroid plexus; PVA, paraventricular thalamic nucleus; VL, ventrolateral thalamic nucleus; Pir, piriform cortex; SON, supraoptic nucleus; SCh, suprachiasmatic nucleus; PVNp, paraventricular hypothalamic nucleus, parvocellular part; PVNm, paraventricular hypothalamic nucleus, magnocellular part; DR, dorsal raphe nucleus; LC, locus coeruleus; Me5, mesencephalic trigeminal nucleus.C: N-CoR/SMRT ratio in selected glucocorticoid areas in individual rats (n= 4). The order of the brain nuclei is arbitrary and the lines drawn in-between is for visualisation purposes only. Asterisks indicate significantly different ratios compared to all other regions.

To compare the respective levels of nuclear N-CoR and SMRT protein, we calculated (in a similar manner as described for the relative mRNA expression analysis) the relative immunoreactivity in 5 selected glucocorticoid and/or mineralocorticoid receptor expressing

brain regions involved in the regulation of the HPA axis (fig. 4c). The ratios of N-CoR/SMRT follow the same pattern for each individual rat. The DG is a significantly N-CoR-enriched brain region compared to the other areas studied (NCoR/SMRT ratios approaching 1.5). Similarly, the LC is significantly SMRT-enriched brain region. The CA3, PVNp and DR cells did not significantly differ in their relative N-CoR and SMRT nuclear protein abundance (ratios approximately 0.8).

3.3 Nuclear distribution of N-CoR and SMRT

Colocalisation of both corepressor proteins within the nucleus was demonstrated by confocal microscopy. Representative pictures of N-CoR and SMRT colocalisation in CA1 hippocampal neurons are shown in fig. 3g-i. The quantification of the nuclear N-CoR-ir and SMRT-ir of all areas studied was performed using immunofluorescence microscopy, i.e. whole cell nuclear signal. Merged confocal picture of N-CoR-ir (green) and SMRT-ir (red) is shown (fig. 3i). The yellow coloring indicates that both proteins colocalise in the CA1 neurons. In general, we found that N-CoR and SMRT proteins colocalise in all areas studied (e.g. hippocampus, PVN, DR, LC) and that their nuclear distribution was speckled. Although N-CoR and SMRT were shown to colocalise in the nucleus of all the cells, nuclear agglomerates containing exclusively N-CoR or SMRT were also observed (data not shown).

4. Discussion

In the present study we examined the neuroanatomical distribution of two functionally distinct corepressors involved in the regulation of gene expression by nuclear receptors. N-CoR and SMRT mRNAs were found to be ubiquitously expressed in brain tissue of young adult male rats. Brain areas such as the cortex, caudate putamen and nucleus accumbens, displayed similar respective levels of expression of both mRNAs. Distinct differences in relative expression were found in discrete regions of the hippocampus, hypothalamus, thalamus and brainstem. We validated this uneven distribution found for both mRNAs at the protein level in glucocorticoid sensitive brain areas that are involved in the regulation of stress-induced HPA-axis activity, i.e. hippocampus, PVN and LC. Furthermore, specific cytoplasmic N-CoR staining was detected in pyramidal cells of cortical areas and the hippocampus. Our results by confocal microscopy indicated that N-CoR and SMRT colocalise *in vivo*, possibly reflecting complexes identified *in vitro* by biochemical purification from HeLa nuclear extracts (13). However, a difference in nuclear distribution of the corepressor proteins (data not shown) was also observed, indicating that even if both proteins associate in homologous complexes, they also are expected to be exclusively present in other nuclear complexes. Taken together, these data reinforce the concept that the uneven distribution of coregulator proteins such as N-CoR and SMRT may have implications for the modulation of site-specific effects of nuclear receptors in brain tissue.

So far, N-CoR and SMRT mRNA expression in rodent brain has primarily been studied in relation to thyroid hormone in juvenile animals (14,15). In an elegant paper, Becker *et al.* also reported an uneven distribution on N-CoR and SMRT mRNA in the hypothalamus of 22 days old mice. Furthermore, in line with our findings, at postnatal day 1, N-CoR transcript was reported to be expressed at high levels in thalamus while SMRT transcript was abundant in the PVN. The parallel expression of N-CoR and SRC1 was also characterised in the fetal and neonatal developing rat brain (14).

While the corepressors have been studied almost exclusively in relation to nuclear processes, we observed cytoplasmic expression of N-CoR in the cortical areas and hippocampus.

Previously, Boutell *et al* convincingly demonstrated using the same antibody as used in our experiments, cytoplasmic localisation of N-CoR in healthy and diseased human brains (16). These authors reported that N-CoR specifically binds to the rat and human Huntington gene product. Although the functional relevance of this interaction remains elusive, these findings and the data in the present study, suggest an additional role of this corepressor protein, apart from its function in nuclear receptor signalling.

The uneven distribution of N-CoR and SMRT suggests distinct physiological roles for both proteins. Assessment of their function on gene transcription was performed in numerous *in vitro* studies. Ligand-activated thyroid hormone receptors transcriptional activity on a well-characterised negative hormone responsive element, showed that SMRT but not N-CoR surprisingly functioned as a coactivator of gene transcription (17). Although Berghagen *et al.* concluded that the hormone response element architecture was important for the function of the corepressor (SMRT), the type of ligand and the amount of corepressor (N-CoR) cotransfected was also found to differentially affect the transcriptional activity of the androgen receptor in CHO cells (18). Additional support for distinct biological roles for N-CoR and SMRT comes from the N-CoR knockout mice which die before birth, indicating that SMRT can not compensate for the lack of N-CoR. Furthermore, the knockout phenotype underlined the importance of N-CoR for at least the development of the nervous system (19).

In our particular interest are the effects elicited by glucocorticoid hormones in the brain. Glucocorticoids bind to two corticosteroid receptors that are widely expressed in the brain, and N-CoR and SMRT expression is high in many areas known to contain MR and/or GR (20-22). In the cells expressing both receptor types, mainly in limbic areas, the MRs and the GRs act in synergism or antagonism on gene transcription, depending on cellular context (23,24). The relative abundance of MR and GR in a cell is a crucial component for the type of effects on gene transcription elicited by the glucocorticoids. However, differences in MR and GR neuroanatomical distribution do not satisfactorily explain cell-specific effects elicited by glucocorticoids on several target genes (corticotrophin releasing factor, phenylethanolamine N-methyltransferase, tyrosine hydroxylase) (25,26).

Our finding that the LC and the substantia nigra differ in their relative N-CoR and SMRT mRNA abundance may be of relevance. The LC was found to be a SMRT-enriched brain area whereas the substantia nigra (SN) expresses both transcripts (see table 1). It is known that chronic cold exposure significantly reduces mRNA expression of the tyrosine hydroxylase (TH) gene (27), the rate-limiting catecholamine biosynthesising enzyme, in the LC whereas it does not affect TH expression in the SN (28,29). In addition, a functional glucocorticoid responsive element has been identified in the 5' flanking promoter region of the TH gene, suggesting that the effects are mediated through DNA-binding of the GR in the promoter region (30,31). Our finding of an uneven expression of N-CoR and SMRT mRNA in brain areas such as in the LC and the SN might in part account for the cell-specific effects of glucocorticoids on TH gene regulation. In the suprachiasmatic nucleus, where no detectable amounts of corticosteroid receptors are expressed, both N-CoR and SMRT are detected (see table 1) indicating that these corepressors are functionally not restricted to the modulation of the transcriptional activity of the corticosteroid receptors.

This uneven distribution of the expression observed for N-CoR (the most striking difference with SMRT has been observed in the brain stem where hardly any detectable amounts were found) may originate from the initial patterning of the central nervous system. Mechanisms such as 'early neural patterning' or 'neural induction' are involved in the subdivision of the neural plate into distinct territories: forebrain, midbrain, hindbrain and spinal cord (for review

(32)). Possibly, this could account for the regional expression of N-CoR reported in our study. Interestingly, the neuroanatomical distribution reported for the coactivators SRC1a and SRC1e by Meijer et al. (2) supply additional support for the proposed explanation, although the expression of the splice variants is presumably governed by a more complex set of genes. Therefore, we speculate that major brain subdivisions might differ in their steroid sensitivity due to a differential expression of transcription coregulators such as N-CoR, SMRT, SRC1a and SRC1e.

The concept that the relative abundance of coregulators in vicinity of promoter regions contributes to cell-specific responses on gene transcription is based on *in vitro* studies. Therefore, the differential distribution of coregulator proteins still has to be examined for its biological relevance. We propose that presented data in combination with the steroid receptor coactivator mapping may offer an explanation and possibly a prediction of the nature of the steroid receptors mediated effects on gene transcription.

5. Acknowledgments

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Chapter III

Nuclear Receptor Coregulators Differentially Modulate Induction and Glucocorticoid Receptor-Mediated Repression of the Corticotropin-Releasing Hormone Gene

S van der Laan, SB Lachize, E Vreugdenhil, ER de Kloet and OC Meijer

Abstract

Nuclear receptor coregulators are proteins that modulate the transcriptional activity of steroid receptors, and may explain cell specific effects of glucocorticoid receptor action. Based on the uneven distribution of a number of coregulators in corticotropin-releasing hormone (CRH) expressing cells in the hypothalamus of the rat brain, we tested the hypothesis that these proteins are involved as mediators in the glucocorticoid induced repression of the CRH promoter. Therefore, we assessed the role of coregulator proteins on both induction and repression of CRH in the AtT-20 cell line, a model system for CRH repression by glucocorticoids. The steroid receptor coactivator 1a (SRC1a), SRC-1e, nuclear corepressor (N-CoR) and silencing mediator of the retinoid and thyroid hormone receptor (SMRT) were studied in this system.. We show that the concentration of glucocorticoid receptor and the type of ligand, i.e. corticosterone or dexamethasone, determines the repression. Furthermore, overexpression of SRC1a, but not SRC1e, increased both efficacy and potency of the glucocorticoid receptor mediated repression of the forskolin-induced CRH promoter. Unexpectedly, co-transfection of the corepressors N-CoR and SMRT did not affect the corticosterone-dependent repression, but resulted in a marked decrease of the forskolin stimulation of the CRH gene. Altogether, our data demonstrate that 1) the concentration of the receptor, 2) the type of ligand and 3) the coregulator recruited, all determine the expression and the repression of the CRH gene. We conclude that modulation of coregulator activity may play a role in the control of the hypothalamus-pituitary-adrenal axis.

1. Introduction

Corticotropin-releasing hormone (CRH) is critically involved in regulation of the hypothalamus-pituitary-adrenal axis (HPA-axis) activity and in diverse stress related behavioural responses involving fear and anxiety (1). The 41 amino acid long neuropeptide is expressed in multiple brain areas (2;3), and regulation of its expression is considered crucial for appropriate adaptation to stressors (4). 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 (CeA). Accordingly, adrenalectomy has the opposite effects on CRH gene expression in the CeA and PVN (5). The molecular mechanism by which the glucocorticoid receptor (GR) simultaneously mediates opposing effects on the same promoter, dependent on the cellular context, so far remains unknown.

Numerous coregulator proteins interact with nuclear receptors such as the GR to regulate the transcription of target genes (6;7). The interactions of these proteins with the DNA-bound steroid receptor form an essential mechanism in the modulation of the genomic response. Coregulators are typically divided in two different classes, coactivators and corepressors (8). The coactivator proteins such as members of the well-documented family of p160 steroid receptor coactivator (SRCs) possess intrinsic histone acetyltransferase activity (HAT) and contain distinct regions and motifs for the recruitment of other proteins with enzymatic activities, including CREB-binding protein (CBP)/p300 and coactivator-associated arginine methyltransferase 1 (CARM-1). Acetylation of histones is important in maintaining an open chromatin structure, thereby facilitating the access of the ligand-activated GR to the proximal promoter of a target gene (9). Conversely, corepressors such as N-CoR (nuclear corepressor) and SMRT (silencing mediator of the retinoid and thyroid hormone receptor) proteins have intrinsic histone deacetyltransferase activity (HDAC) that catalyses the deacetylation of the chromatin. They form docking surfaces for the recruitment of additional components of corepressor complexes, resulting in a reduction of the transcriptional activity (10). The importance of coregulators for GR-mediated transcriptional regulation has been studied mainly in the context of transactivated target genes. The ratio of coactivators and corepressors expressed in the cell has been proposed to determine the nature and the magnitude of the GR-mediated transcriptional response, particularly at subsaturating levels of corticosterone (11).

The activity of coregulators depends on their expression levels as well as posttranslational modifications of the proteins. Recently, we have mapped in the rat brain the expression of the coregulators SRC1a, SRC1e, N-CoR and SMRT (12;13), which are all known to interact with the ligand-activated GR *in vitro* (14;15). These coregulators are abundantly expressed in brain tissue and have distinct expression patterns. In line with the proposed models of steroid action, we hypothesised that their different distribution in brain and their specific contribution to nuclear receptor activity possibly define in part the cell-specific responses elicited by glucocorticoids. The most compelling observation in this respect is that SRC1a is highly abundant in the PVN, coinciding with the site specific glucocorticoid-dependent repression of the CRH gene (16).

A well-established model to study GR mediated repression of the CRH gene is the AtT-20 cell-line (17). The proximal promoter of this gene contains among others an inducible cyclicAMP responsive element (CRE) and a negative glucocorticoid responsive element (nGRE). To characterize the role of the coregulators in both forskolin-induction and glucocorticoid-dependent repression of the CRH gene, we performed transient transfections in AtT-20 cells. We show that SRC1a (but not SRC1e) specifically enhances GR-mediated repression at physiologically relevant concentrations of the naturally occurring ligand corticosterone. In

addition, corepressors do not affect gene repression by GR, but reduce CREB-mediated CRH expression.

2. Materials and Methods

2.1 Plasmids

The pCRH(-918) luciferase reporter was kindly provided by dr. R.I. Dorin (Albuquerque Veterans Administration Medical Centre, New Mexico, USA). Expression plasmids pCMX-N-CoR and p-CMX-mSMRTa were kindly provided by respectively dr. M.G. Rosenfeld (Howard Hughes Medical Institute, USA) and dr. R.M. Evans (Howard Hughes Medical Institute, USA). As a positive control for CREB activation the 5XCRE-TATA-pgl2 reporter plasmid was used (kindly provided by T. Schouten, Leiden University Medical Centre). The expression plasmids of wild type SRC1 splice variants and rGR were previously tested and described (14;18).

2.2 Small interference RNA constructs

Small interference RNA (siRNA) expression vector and mismatch control directed against the consensus sequence of human, rat and mouse GR were designed. The sequence for siRNA against mouse GR (**NM_008173**) was:
GATCCCCGAAAGCATTGCAAACCTCATTCAAGAGATGAGGTTTGCAATGCTTTCT TTTTGGAAA for the perfect match and GATCCCCGACAGCATTGCACACCTCATTCA AGAGATGAGGTGTGCAATGCTGTCTTTTTGGAAA for the mismatch control. The sense and antisense oligonucleotides of 64 bp long were annealed and cloned in *Bgl*II and *Hind*III sites of p-super vector (Netherlands Cancer Institute, Amsterdam, The Netherlands). Insertion of the oligonucleotides was confirmed by sequencing. The knock-down of the GR was tested by western blot and qPCR in rat PC12 cells (Van Hooijdonk L., Brinks V., Meijer O.C., De Kloet E.R., Schouten T., Dijkmans T.F., Vellinga A.C.A., Oitzl M.S., Fitzsimons C.P. and Vreugdenhil E. in preparation) and functionally tested in AtT-20 cells on a 3x containing GRE reporter TAT-3-luc.

2.3 Cell culture and transient transfections

AtT-20/D-16V mouse tumor cells (kindly provided by dr. J. van der Hoek, Erasmus Medical Centre, Rotterdam, The Netherlands) were grown and maintained in DMEM containing 4.5 g/l glucose supplemented with 0.5 % penicillin/streptomycin, 10% horse serum and 10% fetal bovine serum (Invitrogen, Breda, The Netherlands) in a humidified atmosphere of 5% CO_2 at 37°C. A day prior to transfection 0.1×10^6 cells per well were plated in 24 wells plate (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). For each well, the cells were transfected using 1,6µl Lipofectamine 2000 (Invitrogen, Life technologies, Breda, The Netherlands) per 0,8µg plasmid according to the manufacturer's instructions. To induce the CRH-promoter the cells were treated with 10µM forskolin (Calbiochem, Darmstadt, Germany) which leads to an increase of intracellular cyclicAMP (cAMP). Subsequent protein kinase A (PKA) activation results in CREB phosphorylation (19). Repression of the forskolin-induced CRH promoter was performed with the synthetic glucocorticoid dexamethasone (DEX) or the naturally occurring glucocorticoid corticosterone (CORT) co-treatment. The cells were harvested and assayed according to the luciferase kits instructions (Promega, Madison, USA) using a luminometer (LUMAT LB 9507, Berthold, Bad Wildbad, Germany).

First, we further validated the AtT-20 cells as a model for glucocorticoid-dependent repression of the CRH promoter. For the GR knock-down, 400ng of siRNA expression plasmid, 200ng of CRH reporter and 200ng of empty vector plasmid were transfected. After transfection,

the cells were allowed to recover for 48 hours prior to treatment. For the dose response curves of dexamethasone and corticosterone, serial 10x dilutions were prepared from a 10^{-6} M solution. To characterise the role of the coregulators, 200ng of reporter, 400ng of expression and 200ng of empty vector plasmid were transfected per well. For the dose-response curves of corepressors, 200ng of reporter and a varying amount of corepressor expression plasmid were transfected. The total amount of DNA for each transfection was kept constant using empty vector.

2.4 Data analysis

Two different renilla reporters were tested (pRL-TK and pRL-CMV) for normalisation. Both were found responsive to forskolin and dexamethasone treatment in AtT-20 cells (data not shown). Consequently, the data were normalised against basal activity of the CRH-promoter (fold induction) or expressed as percentage of maximal induction of the CRH-promoter after forskolin stimulation (% of maximal induction).

2.5 Statistics

The values are expressed as the average of 4 paralleled transfections within one experiment and the error bars represent the standard deviation. All transfection experiments were performed at least twice, yielding similar results. Overall statistical analysis was performed using one way analysis of variance (ANOVA) and statistical significance was determined with Tukey's multiple comparison tests with $p < 0.05$.

3. Results

3.1 CRH-promoter activity and glucocorticoid-induced repression in AtT-20

Forskolin treatment for 4 and 24 hours led to a marked increase of the CRH-promoter activity in transiently transfected AtT-20 cells (93± 6 and 68± 8 fold induction at respectively 4 and 24 hours). The sustained 24 hours treatment resulted in a lower luciferase signal compared to the 4 hours treatment. Furthermore, co-treatment with 10^{-7}M dexamethasone (DEX) strongly reduced the forskolin induced stimulation of the CRH-promoter in both groups. The repression caused by the co-treatment with 10^{-7}M DEX was 66% at 4 hours and 44% at 24 hours (fig 1A). Unless stated otherwise, all subsequent data were generated after 4 hours of treatment, the time point at which most robust DEX-induced repression of the CRH-promoter activity was observed.

In order to confirm the specific role of GR in the DEX-induced repression, and to assess whether the GR is a limiting factor in the present setting, GR was either overexpressed or knocked-down in the AtT-20 cells. The overexpression of the GR protein resulted in a significant increase of the repression induced by DEX (fig. 1B). In this condition, DEX co-treatment resulted in 90% repression, almost completely silencing the forskolin induced stimulation of the CRH-promoter. Conversely, the siRNA mediated knock-down of the GR completely abolished the DEX-induced repression. The mismatch control did not significantly differ from the empty vector group indicating a specific knock-down of the GR.

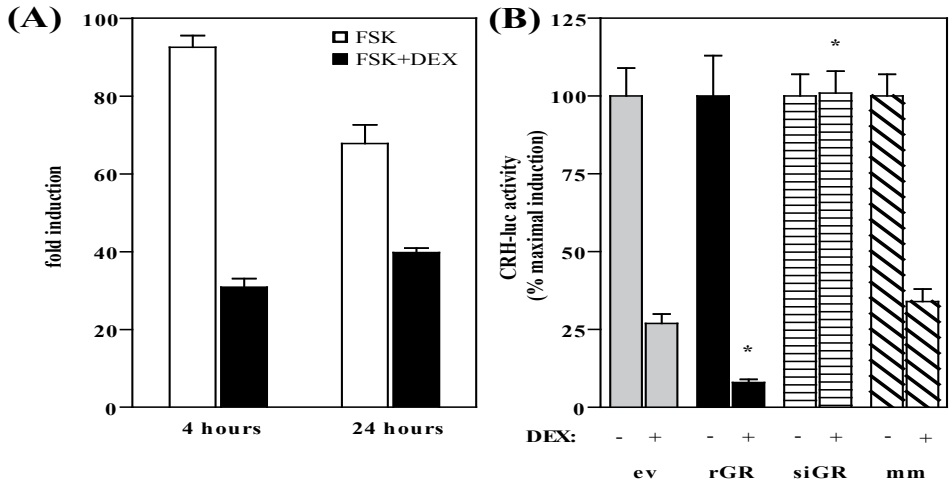

Fig. 1: GR-mediated repression of the forskolin-induced CRH-promoter activity.
(A) Dexamethasone-induced CRH repression after 4 and 24 hours co-treatment with forskolin. Data are expressed as the fold induction over basal activity of the promoter in untreated cells. The average values (n=4; ±SD) are shown. The DEX-induced repression of the CRH-promoter was higher after 4 hours of incubation compared to 24 hours. (B) Role of the GR in the dexamethasone-induced repression. The CRH-promoter activity was measured after 4 hours of incubation and normalised against the maximal effect of forskolin without steroid in each group (ev: empty vector, rGR: rat GR expression plasmid, siGR: siRNA against GR, mm: mismatch siRNA control). The average values (n=4; ±SD) are shown. * indicates significantly different from the repression induced by dexamethasone in the empty vector group ($P<0.001$). The level of repression of the CRH-promoter activity is GR dependent.

These data together indicate that the concentration of GR protein in the AtT-20 cells determines the magnitude of the DEX-induced CRH repression. The present setting forms a suitable model to assess coregulator protein function in the GR-mediated repression of the CRH-promoter activity.

3.2 Dexamethasone and corticosterone induced CRH repression

The aim of our study was to address whether brain-expressed coregulator proteins play a role in the regulation of the CRH gene expression. While it is known that the GR activity depends on the type of ligand (20), so far, the synthetic glucocorticoid dexamethasone has been the treatment of choice in most studies on glucocorticoid mediated CRH repression in AtT-20 cells (21-23). In order to approach to a maximal extent the *in vivo* situation, we tested whether the naturally occurring ligand of the GR in rodents, i.e. corticosterone, resulted in similar repression of the CRH gene. We generated a time curve of CRH-promoter activity, and compared the repressive effects of 10^{-7}M DEX with 10^{-7}M CORT co-treatment (fig 2A). Both the natural and the synthetic glucocorticoid suppressed CRH-promoter activity, although not to the same extent. A maximal repression of 40% and 70% was achieved after 4-5 hours with 10^{-7}M CORT and 10^{-7}M DEX co-treatment, respectively.

Additionally, we generated dose-response curves of DEX and CORT co-treatment for the GR-mediated repression of the CRH-promoter activity. As shown in figure 2B, both efficacy and potency were higher for DEX co-treatment compared to CORT co-treatment. The effective concentration of DEX ($EC_{50\,(DEX)}$) was 0.46± 0.004 nM and the maximal repression of 75% was achieved at 10^{-7}M. Co-treatment with CORT was less effective and the maximal

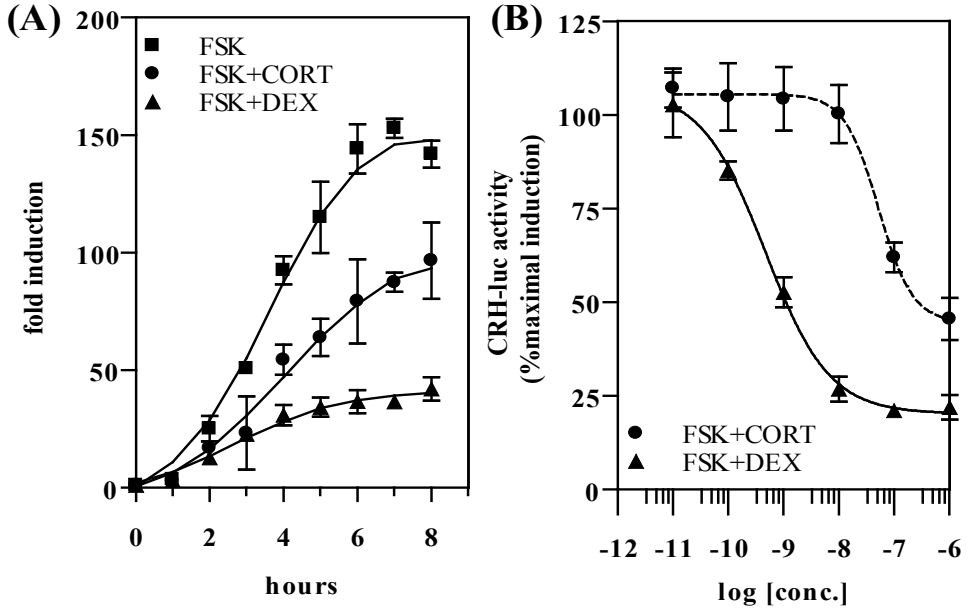

Fig. 2: Corticosterone-dependent repression of the CRH-induced gene.
(A) Time course of CRH-promoter activity after forskolin treatment w/wo dexamethasone or corticosterone co-treatment. The effect of forskolin (■), co-treatment with 10-7M DEX (▲) or 10-7M CORT (▼) on CRH-promoter activity are shown over 8 hours. The average values (n=4; ±SD) are shown and represent the fold induction. (B) Dose-response curve of dexamethasone and corticosterone co-treatment. The luciferase activity at each steroid concentration was measured after 4 hours incubation and normalised against the maximal effect of forskolin without steroid. The average values (n=4; +/-SD) are shown and represent the fold repression mediated by the two different co-treatments on CRH-promoter activity. Dexamethasone is more potent in suppressing CRH-promoter activity.

repression of 55% was observed at 10^{-6}M CORT co-treatment. This was significantly lower compared to the DEX-induced maximal repression. The EC_{50} of the CORT-induced repression ($EC_{50(CORT)}$) was =52 ± 1 nM, approximately 100 times higher than for DEX co-treatment.

3.3 Differential effects of SRC1 isoforms on GR-mediated CRH repression

In the next experiments we tested the effect of SRC1a and SRC1e overexpression on GR-mediated repression of the CRH-promoter activity. Co-transfection of SRC1a resulted in a significant GR-mediated repression at 10^{-8}M CORT (fig. 3B). The potency of CORT to induce repression was approximately 3 times higher in the presence of SRC1a ($EC_{50(SRC1a)}$ =18±0.3nM) when compared to the control situation ($EC_{50(CORT)}$ =52± 1nM). On the other hand, overexpression of SRC1e tended to shift the dose-response curve to the right, and resulted in a 2 times higher EC_{50} ($EC_{50(SRC1e)}$=109±0.3 nM) compared to the control group (fig. 3C). These data indicate that SRC1a and SRC1e have opposing effects on the CORT-induced repression (fig. 3D). Moreover, the maximal repression by GR in presence of both SRC1 isoforms significantly differed from the control group. SRC1a overexpression resulted in a maximal repression of 67%, which was higher than both the maximal repression in the control group (max. repression =55%) and in presence of SRC1e (max. repression =45%). In summary, both the efficacy and potency of the GR-mediated repression of the CRH-promoter were higher in presence of SRC1a and tended to be reduced in presence of SRC1e.

3.4 N-CoR and SMRT do not affect GR-mediated CRH repression

Subsequently, we tested whether corepressor expression would affect the GR-mediated repression of the CRH-promoter. Interestingly, forskolin treatment led to a reduced maximal induction of the CRH gene expression when N-CoR or SMRT were co-transfected (fig.4B and 4C), suggesting that CREB-directed transcription of the CRH gene is suppressed by both corepressors (30-fold compared to a typically 100-fold as observed in absence of overexpressed coregulators, fig 4A). Surprisingly, N-CoR and SMRT overexpression resulted in a similar dose-response curve for transrepression compared to the control situation (fig 4D). A maximal repression of approximately 55% for both situations was observed at 10^{-6}M CORT and a highly similar potency. The effective concentration in presence of N-CoR or SMRT was $EC_{50(N\text{-}CoR)}$ =47± 1nM and $EC_{50(SMRT)}$ =49± 1nM respectively. Although both corepressors have been shown to interact with agonist-activated GR on positively regulated genes (15;24), they do not modulate the GR-mediated repression of the CRH-promoter at the nGRE. The effects of the coactivators and the corepressors on the GR-mediated repression of the CRH-gene expression are summarised in table 1.

	Control	SRC1a	SRC1e	N-CoR	SMRT
Maximal repression at 10^{-6} CORT	55%	67% (*)	45% (*)	55%	55%
EC_{50} CORT (nM)	52 ± 1	18 ± 0,3	109 ± 0,3	47 ± 1	49 ± 1

Table 1: Effect of coregulator overexpression on the GR-mediated repression of the CRH-promoter. The control group was co-transfected with the empty vector plasmid. Maximal repression at 10-6M CORT co-treatment is expressed as percentage of the forskolin-induced CRH-promoter activity. * indicates significantly different from the maximal repression in the control group ($p<0.05$). EC50 is the effective concentration CORT at which 50% of the maximal repression is achieved.

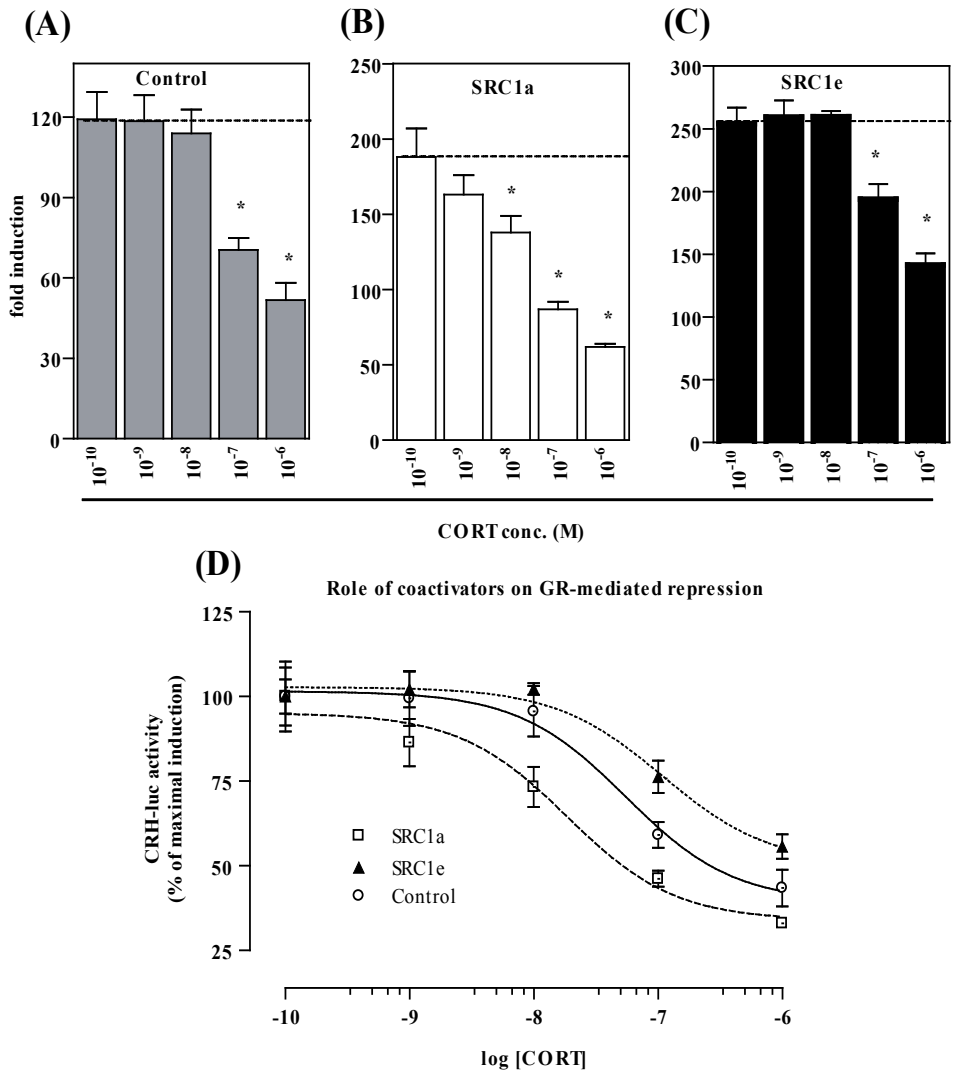

Fig. 3: Differential effect of SRC1 isoforms on the GR-mediated CRH repression.
CRH-promoter activity expressed as fold induction (normalised against basal activity of the promoter). The luciferase activity was measured after 4 hours incubation. The average values (n=4; ± SD) are shown. * indicates significantly different from the group with 10-10M corticosterone ($P<0.001$). (A) The control group was co-transfected with the empty vector plasmid. (B) Steroid receptor coactivator splice variant SRC1a modulates the CORT-induced repression of the CRH-promoter. Overexpression of SRC1a resulted in a significant repression at 10-8M CORT. (C) SRC1e overexpression resulted in a significant repression at 10-7M CORT. (D) CORT-induced repression as percentage of maximal induction by forskolin. Co-transfection of SRC1a results in a left-shift of the dose response curve.

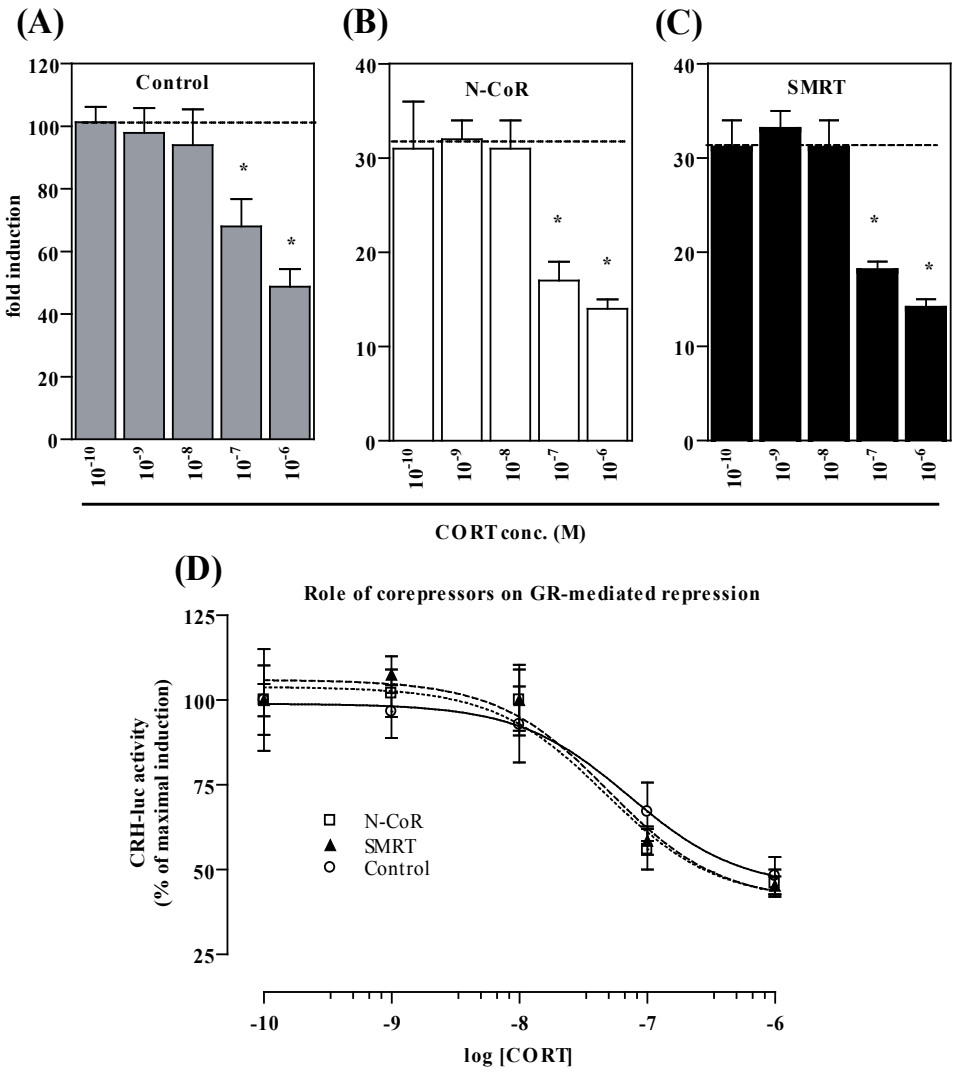

Fig. 4: Corepressors N-CoR and SMRT do not affect the GR-mediated CRH repression.
CRH-promoter activity expressed as fold induction (normalised against basal activity of the promoter). The luciferase activity was measured after 4 hours incubation. The average values (n=4; ± SD) are shown. * indicates significantly different from the group with 10-10M corticosterone ($P<0.001$). (A) The control group was co-transfected with the empty vector plasmid. Overexpression of the corepressors N-CoR (B) and SMRT (C) suppresses the forskolin-induced promoter activity. (D) Neither N-CoR nor SMRT changes the dose-response curve of corticosterone. This suggests that N-CoR and SMRT repress CREB activity but do not interact with the GR-mediated repression.

3.5 N-CoR and SMRT dose-dependently suppress CREB-mediated transcription

To further examine the role of the corepressors N-CoR and SMRT on CREB-mediated transcription, we compared the effect of increasing amounts of the corepressors on two different CRE-containing reporter constructs, i.e. the composite CRH-promoter and the simple 5xCRE-containing promoter (fig. 5A). We found that both N-CoR and SMRT dose-dependently suppressed the CREB-mediated transcription of the CRH-promoter (fig. 5B). On the simple 5xCRE-containing reporter, both N-CoR and SMRT suppressed CREB-mediated transcription even more potently, with a maximal inhibition of the forskolin-induced stimulation after co-transfection of 100ng of expression plasmid (fig. 5C).

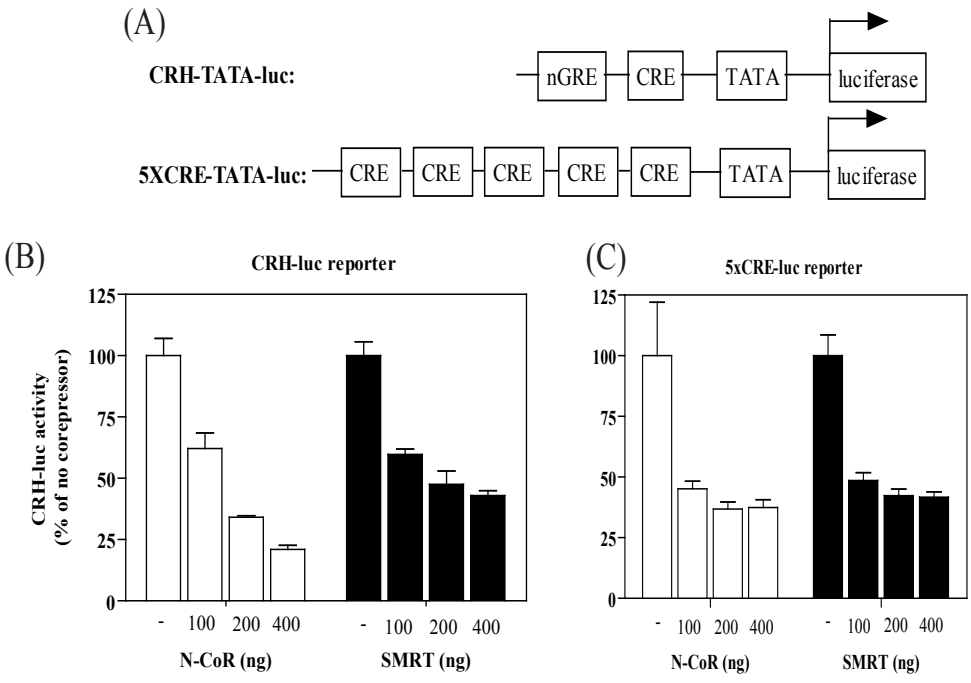

Fig. 5: CREB-mediated transcription is suppressed by N-CoR and SMRT.
(A) Schematic representation of the CRH-luciferase reporter, with both the negative GRE (nGRE) and the CRE, and the synthetic 5xCRE containing reporter. Dose-dependent effects of N-CoR and SMRT overexpression on the CRH-luc reporter (B) or the 5xCRE-luc reporter (C). The luciferase activity was measured after 24 hours forskolin incubation. The average values (n=4; ± SD) are shown.

These data together suggest a direct interaction of CREB with both N-CoR and SMRT at multiple CRE-containing promoters. DEX co-treatment did not affect the FSK induction of the 5xCRE containing promoter, indicating that the GR-mediated repression of the CRH-promoter is not mediated via the cAMP response element (CRE) and or CRE related proteins (fig. 6) but more likely the result of a direct interaction of the GR to the nGRE of the CRH promoter.

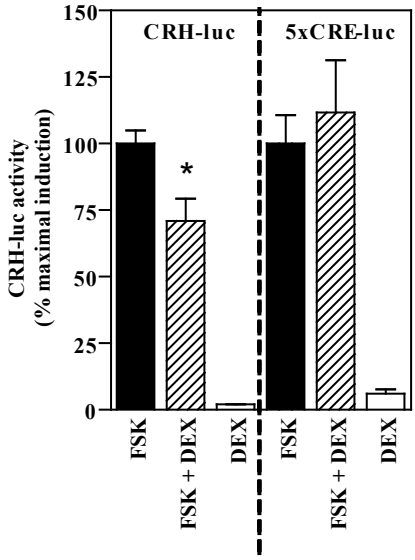

Fig. 6: Promoter specific effects of DEX co-treatment
Co-treatment with 10-7M DEX suppresses forskolin-induced CRH promoter activity whereas it does not affect the promoter activity of the forskolin-induced 5xCRE-containing promoter. The GR-mediated repression is promoter-dependent and is not likely to involve cross-talk with CRE related proteins. The average values (n=4; ± SD) are shown. The luciferase activity was measured after 24 hours treatment. * indicates significantly different from the forskolin-induced promoter activity ($P<0.05$).

4. Discussion

In the present study we tested the hypothesis that the repression of the CRH gene by glucocorticoids is determined by the type of coregulator recruited. The hypothesis is based on the observation that both SRC1 splice variants and corepressors have distinct functional effects on transcription by nuclear receptors, and are differentially expressed in rat brain (12;13). The overexpression of the coactivator SRC1a increased both efficacy and potency of the corticosterone-dependent repression of forskolin-induced CRH expression. Conversely, the corepressors N-CoR and SMRT did not affect the GR-mediated repression but significantly reduced the forskolin stimulation of the CRH-promoter. The data suggest that the high expression of SRC1a in the PVN is likely to be involved in the GR-mediated repression of the CRH gene. Moreover, the expression levels and the modulation of the activity of the coregulators may change the extent of both induction and repression of the CRH gene, as well as other genes that are regulated by similar CREB and GR dependent mechanisms.

Recently, the coactivators SRC1 and SRC2 have been shown to act also as a corepressor of ligand-activated GR at certain GREs (25;26), while on the other hand, the corepressor SMRT has been reported to be essential for full ER activation (27). We have shown that the SRC1a splice variant also acted as a corepressor of ligand-activated GR on the CRH-promoter. Although the mechanisms of repression remain unclear, an increasing amount of literature shows that non-receptor factors such as coregulators determine not only the magnitude, but also the nature of the transcriptional outcome.

So far, coregulator recruitment by ligand-activated GR has mainly been studied in the context of positively regulated genes. In that context, a differential potentiation of the oestrogen

receptor (ER) and GR-mediated transcription by the SRC1 isoforms was previously observed (14;28). We now show that the splice variants also differentially affect the GR-mediated repression of the CRH reporter. The SRC1 splice variants are highly similar. They contain, an identical centrally located nuclear receptor box with a triplet of α-helical 'LXXLL' motifs for binding to the nuclear receptors, two activation domains, AD1 (interacts with CBP/p300), and AD2 (interacts with CARM1) and differ only in their C-terminal sequences. The C-terminal part of SRC1a contains an additional LXXLL motif which was found to exhibit a strong interaction with the ligand binding domain (LBD) of the GR in a yeast two-hybrid system (29). Additionally, it also has been shown to possess a repression domain in the context of ER and GR-mediated gene transactivation on a simple reporter (14;28). Therefore, the differences in transrepression may be caused either by differential recruitment of SRC1a and SRC1e to the DNA-bound GR, and/or by the SRC1a specific C-terminal repression domain.

It is still a topic of debate whether the GR-mediated repression on the CRH promoter occurs via the cAMP response element (CRE) and/or CRE-related proteins (e.g. CBP), via cross-talk with AP1, or by direct binding of the GR to a putative negative GRE in the promoter region (17;21;23;30). In the neuronal BE(2)C cell-line, point mutation and deletion of the putative nGRE did not affect dexamethasone-induced repression (30). However, Dorin *et al.* convincingly showed that the putative nGRE in the AtT-20 cell line is necessary for GR-mediated repression of the CRH-promoter (21). Additionally, an argument against interaction of GR with the CREB signalling pathway (on or off the DNA) is our observation that forskolin stimulation of a simple reporter containing 5xCRE was not affected by DEX co-treatment in the same cells (fig 6). Furthermore, taking into consideration the sequential and combinatorial assembly of protein complexes at the promoter by DNA-bound steroid receptors (31;32), the effects of SRC1a suggests that the binding of GR to the response element of the CRH gene is necessary. We propose that the GR-mediated repression of the CRH gene expression is promoter specific, and cannot be explained by squelching of rate limiting factors such as CBP or CRE related proteins.

Both CRH and POMC genes can be directly repressed by GR and are part of the negative feedback regulation of glucocorticoids on the HPA-axis activity. Interestingly, the 5' flanking region of both CRH and the POMC genes contain a putative negative GRE (nGRE) (21;33). Winnay *et al.* reported that, as opposed to the situation in wild type mice, dexamethasone was ineffective in suppressing pituitary POMC mRNA levels in the SRC1 knock-out mice. This indicates that SRC1 expression is necessary for adequate GR-mediated repression of the POMC gene (34). These observations suggest that the glucocorticoid repression of the HPA-axis activity at the level of the hypothalamic CRH and pituitary POMC expressing cells, are both dependent on SRC1a recruitment by the GR at a nGRE.

The corepressors N-CoR and SMRT are ubiquitously expressed in brain and show moderate differences in expression in the PVN (13). Previously, Szapary *et al.* established a model based on the observations that coactivators and corepressors have opposing effects on the dose response curve of agonist bound GR regulated gene expression (11). The model describes that the GR dose-response curve can be modified by coactivators and corepressors in a gene and promoter independent fashion. Surprisingly, our data showed no effect of the corepressors N-CoR and SMRT on the GR-mediated repression of the CRH gene. We find that coactivators and corepressors did not mediate opposing effects on a negatively regulated gene, indicating a clear promoter-dependent effect of coactivators and corepressors. It is likely that the binding of the agonist-activated GR to the nGRE induce conformational changes that disfavour corepressor recruitment.

Although N-CoR and SMRT did not affect the GR-mediated repression, they both repressed CREB-mediated induction of the CRH gene. This is in line with the observation that N-CoR can directly modulate the activity of the cointegrator CBP by binding to the same complexes (35). Interestingly, although N-CoR and SMRT can associate with distinct corepressor complexes, overexpression resulted in a very similar inhibition of CREB-mediated transcription (36). We examined promoter specific effects of the corepressors, and tested overexpression of N-CoR and SMRT on CREB-mediated transcription on the CRH-luc reporter and a simple 5xCRE containing reporter. Both corepressors dose-dependently suppressed the CREB-mediated transcription, clearly indicating that the effects are mediated through a similar mechanism (fig. 5).

Taken together, coregulators may play different roles in the control of hypothalamic CRH expression. The brainstem catecholaminergic system projects to the PVN and releases norepinephrine that binds to the G-protein coupled α1 adrenergic receptors and activates CREB-mediated gene expression. Additionally, glutamatergic and GABAergic interneurons also regulate the activity of the PVN and in turn expression of CRH (37-41). The fact that N-CoR and SMRT are expressed in the PVN, and our present finding that they inhibit the CREB-mediated transcription of the CRH gene, indicate that the corepressor activity may in part determine the CRH gene expression. In addition, Shepard *et al.* recently provided evidence for the role of cAMP response element (CRE) modulator (CREM) and inducible cAMP early repressor (ICER) in limiting the CRH surge in the context of restraint stress (42). Thus, repression of the CRH gene expression after stress is likely to involve a set of different mechanisms. We provide evidence that coregulator proteins have specific roles in both induction and repression of CRH gene expression.

Paradoxically, glucocorticoids repress CRH expression in the PVN but stimulate expression in the central nucleus of the amygdala (CeA) (5). The current data are based on overexpression of coregulators: the exact ratio of receptor to coregulators is difficult to ascertain. However, the data provide evidence that SRC1a, highly abundant in the PVN, increases both potency and efficacy of the GR-mediated repression of the forskolin-induced CRH expression. In addition, SRC1e which was previously found to be relatively abundant in the CeA (12), significantly reduced the corticosterone-dependent repression (see table 1). Moreover, SRC1e tended to increase FSK-induced CREB-driven transcription of the CRH gene (fig 3C). These observations are in line with the site-specific effects of glucocorticoids on CRH gene expression previously described *in vivo*. In view of the chromatin modifying properties of the coregulators, further functional studies in stably transfected AtT-20 cells and chromatin immunoprecipitation assays would provide valuable insights on their role in the regulation of CRH expression.

In conclusion, we have shown that 1) the availability and the type of ligand 2) the expression level of the receptor in the cells and 3) the coregulator recruited are three determinants of the glucocorticoid signalling. More specifically, the coactivator SRC1a increased the GR-mediated repression, while the corepressors N-CoR and SMRT were found to inhibit the CREB-dependent induction of the CRH gene.

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Chapter IV

Timing is critical for effective glucocorticoid receptor mediated repression of the cAMP-induced corticotropin-releasing hormone gene

S van der Laan, ER de Kloet and OC Meijer

Abstract

Glucocorticoid negative feedback of the hypothalamus-pituitary-adrenal axis is mediated in part by direct repression of gene transcription in glucocorticoid receptor (GR) expressing cells. We have investigated the cross talk between the two main signalling pathways involved in activation and repression of corticotrophin releasing hormone (CRH) mRNA expression: cyclic AMP (cAMP) and GR. We report that in the At-T20 cell-line the glucocorticoid-mediated repression of the cAMP-induced human CRH proximal promoter activity depends on the relative timing of activation of both signalling pathways. Activation of the GR prior to or in conjunction with cAMP signalling results in an effective repression of the cAMP-induced transcription of the CRH gene. In contrast, activation of the GR 10 minutes after onset of cAMP treatment, results in a reduced ablitity to repress gene expression. In addition, translocation of ligand-activated GR to the nucleus was found as early as 10 minutes after glucocorticoid treatment. Interestingly, while both signalling cascades counteract on the CRH proximal promoter, they synergize on a synthetic promoter containing 'positive' response elements. Since the order of activation of both signalling pathways may vary considerably *in vivo*, we conclude that a critical time-window exists for effective repression of the CRH gene by glucocorticoids.

1. Introduction

Cross-talk of intracellular signalling pathways is central to many neuroendocrine control systems (1;2). The expression and/or secretion of the two main neuroendocrine secretagogues of the hypothalamus-pituitary-adrenal axis (HPA axis) are both stimulated by cAMP and suppressed by glucocorticoids, the end-product of the HPA axis: adrenocorticotrophic hormone (ACTH) from anterior pituitary corticotrophs and corticotrophin releasing hormone (CRH) from the hypothalamus (3-6). At the molecular level, these signals are represented by protein kinase A (PKA), the transcription factor cAMP element-binding protein (CREB), and the glucocorticoid receptor (GR), respectively.

The proximal promoter of the human corticotrophin releasing hormone (hCRH) gene contains a canonical, functional cAMP response element (CRE) and a negative glucocorticoid receptor response element (nGRE). Induction of the hCRH gene expression by cAMP-dependent activation of the protein kinase A (PKA) pathway is mediated by phosphorylation of the CRE-binding protein (CREB) at serine residue 133 (7;8). *In vivo*, Wölfl *et al.* showed that binding of CREB to the canonical CRE located at the nucleotide position -224 (upstream exon 1) was specifically induced after activation of the PKA pathway with forskolin (9). Additionally, Kovacs *et al.* demonstrated that in the hypothalamic parvocellular neurons of rodents subjected to ether stress, CREB phosphorylation was induced in a time course that parallels the increase of CRH heteronuclear RNA levels (10).

The At-T20 cell-line is a well-established *in vitro* model system for studying glucocorticoid-induced repression of the hCRH proximal promoter. Nested deletions and site-specific point mutations of the CRE located at nucleotide -224 resulted in a significant loss of induction by cAMP, demonstrating that CREB binding is necessary for the stimulation of the gene (11). In parallel, electrophoretic mobility shift assays (EMSA) identified a GR-binding site at position nt -249 that was indispensable for GR-mediated repression of the cAMP-induced promoter. Internal deletion of the entire 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 (12).

The nGRE in the hCRH promoter is separated by as few as 25 bp with the canonical CRE, a distance that clearly permits functional interactions at the promoter (13). Since, *in vivo* the order of activation of the cAMP and glucocorticoid signalling pathways may vary considerably, and this is known to affect responses at the level of neuroendocrine secretion (14), we tested the hypothesis that effective repression of the cAMP-induced hCRH proximal promoter depends on the relative timing of GR activation in the At-T20 cell-line.

2. Material & methods

2.1 Cell culture and transient transfections

AtT-20/D-16V mouse tumor cells (kindly provided by dr. J. van der Hoek, Erasmus Medical Centre, Rotterdam, The Netherlands) were grown and maintained in DMEM containing 4.5 g/l glucose supplemented with 0.5 % penicillin/streptomycin, 10% horse serum and 10% fetal bovine serum (Gibco, United Kingdom) in a humidified atmosphere of 5% CO_2 at 37°C. A day prior to transfection 0.1×10^6 cells per well were plated in 24 wells plate (Greiner). For each well, the cells were transfected using 1.6µl Lipofectamine 2000 (Invitrogen, United Kingdom) per 0.8µg plasmid according to the manufacturer's instructions. To induce the CRH-promoter the cells were treated with 10µM forskolin (Calbiochem, Darmstadt, Germany) which leads to an increase of intracellular cyclicAMP (cAMP). Subsequent protein kinase A (PKA) activation results in CREB phosphorylation (15). Repression of the forskolin-induced

CRH promoter was performed with the synthetic glucocorticoid dexamethasone (DEX) co-treatment. The cells were harvested and assayed according to the luciferase kits instructions (Promega, Madison, USA) using a luminometer (LUMAT LB 9507, Berthold, Bad Wildbad, Germany). The total amount of DNA for each transfection was kept constant using empty vector.

First, GR-mediated repression was measured when both treatments were given simultaneously. Then, to address the hypothesis that the relative timing of activation is of importance we assessed whether GR activation prior to or after the FSK treatment differentially affected the promoter activity of CRH. In these experiments (fig 1B), all groups were treated 3 hours with FSK but the time of onset of DEX treatment relative to FSK varied. Of note, DEX treatment was added at the time mentioned in the figure (relative to start of FSK treatment which was set at t=0). Finally, to test promoter-specificity we used the synthetic TAT3-Luc GRE-containing reporter (fig 3). In the experiments using the GRE-containing promoter, all groups were treated for 4 hours with DEX and the time of onset of FSK treatment varied. If there is no crosstalk between the cAMP/CREB pathway and GR, than the expectation is that all groups have similar promoter activity since the time of DEX treatment is identical.

2.2 Immunocytochemistry

A day prior stimulation, $30x10^3$ cells were grown in chamber slides. Following stimulation, cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100 and blocked with 5% normal goat serum. Cells were incubated with a GR-specific antibody (M20; dilution 1:500; Santa Cruz biotechnologies) during 60 minutes, washed and subsequently incubated for 60 minutes with a secondary goat anti-rabbit Alexa Fluor 488 antibody (dilution 1:750; Invitrogen, Breda, The Netherlands). After incubation, cells were washed and counterstained for 10 min with Hoechst 33528. All sections were mounted with polyaquamount (Polysciences, Inc.) and visualized with an immunofluorescence microscope (Leica DM6000). Control cells were incubated with equal amounts of non-immune rabbit serum (Santa Cruz), which was used as substitute for the primary antibodies. Guided by the Hoechst staining, nuclear immunoreactivity of at least 20 cells was measured using ImageJ 1.32j software (NIH, USA). Nuclear immunoreactivity expressed as relative optical density in Igg-stained controls was used for determination of background signal.

2.3 Data analysis

First, we determined the repression induced by simultaneous DEX/forskolin cotreatment (Figure 1A). Since the aim of the study was to compare the DEX-induced repression on the CRH promoter, we compared all groups to the repression measured in the three hours cotreatment group (set at 100%). In the next experiment (figure 1B) only the onset time of DEX treatment varied between the groups whereas all groups were stimulated for three hours with FSK. The DEX-induced repression is compared.

2.4 Statistics

The values are expressed as the average of 4 paralleled transfections within one experiment and the error bars represent the standard deviation. All transfection experiments were performed at least three times, yielding similar results. Overall statistical analysis was performed using one way analysis of variance (ANOVA) and statistical significance was determined with Tukey's multiple comparison tests with $p < 0.05$.

3. Results and discussion

Three hours forskolin (FSK) treatment strongly induced the CRH-promoter activity. In line with previous reports (12;16), simultaneous DEX cotreatment strongly suppressed the FSK-induced stimulation of the hCRH-promoter activity. DEX cotreatment resulted in 75% repression of the FSK-induced promoter activity (fig. 1A). However, DEX treatment alone (0.1 μM) did not significantly suppress the basal activity of the CRH-promoter (data not shown). To test our hypothesis that the order of activation of both signalling cascades is important for the level of GR-mediated repression, we initiated the DEX treatment at different time points prior or during the 3-hours FSK treatment (fig. 1B). We compared the resulting GR-mediated repression to the simultaneous cotreatment group (to be able to adequately compare repression, the cotreatment group (75%) was set to a 100% reference value). Two hours of DEX pre-treatment resulted in a significant increased repression compared to the simultaneous cotreatment (data not shown). However, activation of the GR up to one hour prior to FSK treatment resulted in similar levels of GR-mediated repression as in the simultaneous cotreatment group (fig. 1B). The relatively slow onset of the additional repression suggests that *de novo* protein synthesis is involved in this effect.

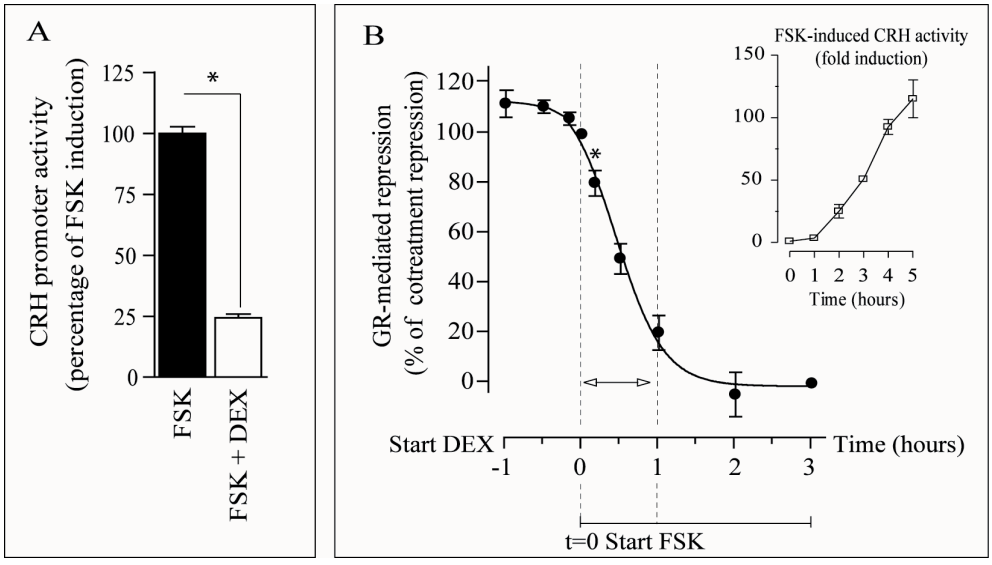

Fig. 1: Luciferase reporter assay in AtT-20 mouse tumour cells. 0.1x106 cells were transiently transfected in 24-wells plate using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The day after transfection, the cells were treated with 10 μM forskolin (Calbiochem, Darmstadt, Germany) and/or 0.1μM of the synthetic glucocorticoid dexamethasone (DEX) and assayed for luciferase activity. (1A) CRH-promoter activity expressed as percentage of maximal induction after 3 hours forskolin (FSK) treatment (filled bar). Simultaneous co-treatment with DEX (open bar) resulted in a strong repression of the CRH-promoter activity. (1B) The repression induced by DEX in the cotreatment group was set at 100%. All groups were treated for three hours with FSK. Different time of onset of the DEX treatment relative to the FSK treatment results in a significant loss of repression when DEX treatment is started 10 minutes after FSK treatment (*). FSK treatment leads to a progressive increase in CRH-luc promoter activity over a period of at least 5 hours (inset).

When DEX treatment is started after forskolin stimulation of the CRH promoter, the time-window separating both treatments was of great consequence for the level of repression (fig. 1B). A 10 minutes delay in DEX treatment resulted in a 20% loss of repression compared to the simultaneous cotreatment group. Strikingly, a 30 minutes delay (a reduction of approximately

15% of the DEX treatment time compared to simultaneous cotreatment) resulted in a 50% loss of GR-mediated repression, indicating the importance of the relative time of onset of treatments. Clearly, the reduced time of DEX exposure is not proportional to the loss of GR-mediated repression, pointing to a 'GR resistance' at the promoter. Because FSK treatment induces a progressive increase of the CRH-luc promoter activity over a period of at least 5 hours (inset fig. 1B), we assume that FSK induces binding of CREB to the promoter over that period. However, activation of the GR in the first hour following FSK treatment is critical for effective repression.

To gain insight in the dynamics of GR translocation to the nucleus, we performed immunocytochemistry on DEX treated cells. Translocation data show that DEX treatment induces maximal nuclear GR-immunoreactivity (GR-ir) as early as 10 minutes after treatment (figure 2). No difference in nuclear GR-ir was observed between the 10 and 30 minutes treatment groups (fig. 2A). As expected, FSK treatment did not influence translocation dynamics of the GR although it is known that PKA activation can modulate the steroid sensitivity by enhancing DNA binding properties of GR (17). These data indicate that GR is capable of meditating its genomic effects as early as 10 minutes after treatment.

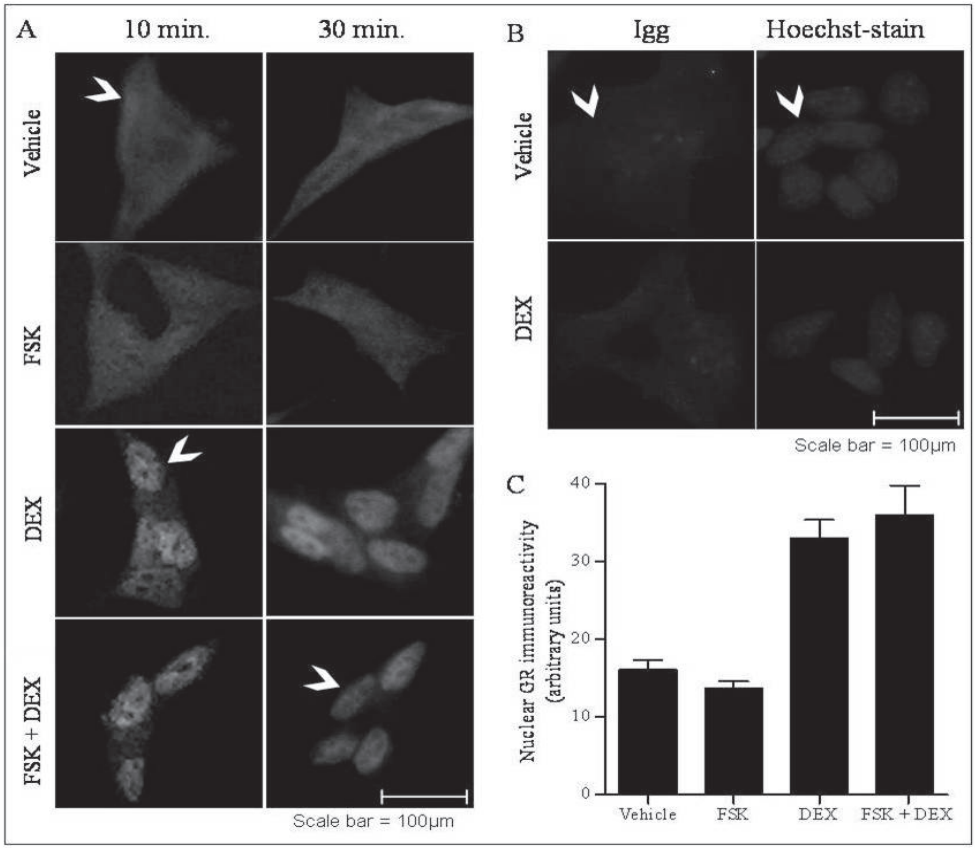

Fig. 2: Immunofluorescent staining of the GR in AtT-20 cells. (2A) Time course of GR-ir in different treatment groups. DEX alone and FSK + DEX cotreatment, show nuclear GR staining after 10 minutes treatment. (2B) Control IgG staining show specificity of the GR-specific antibody. (2C) Nuclear quantification of GR-ir after 10 minutes treatment (The average values ± SEM are shown) (see colour image page 126).

Posttranslational modification such as phosphorylation is known to affect DNA binding properties, transcriptional activation and stability of numerous nuclear receptors including GR (18). Although translocation to the nucleus was not affected by FSK treatment, we tested whether FSK influenced the transcriptional activity of the GR in these cells. We measured the effect of FSK and DEX cotreatment on a positively regulated promoter (a synthetic GRE-containing promoter; TAT3-luc (19)). FSK cotreatment synergistically induced transcription on an exclusively GRE-containing promoter compared to DEX treatment alone (fig. 3). FSK treatment prior DEX treatment resulted in an increased transcriptional activity of the GR. Likewise, the longer the time of FSK cotreatment the higher the transcriptional activity of the GR.

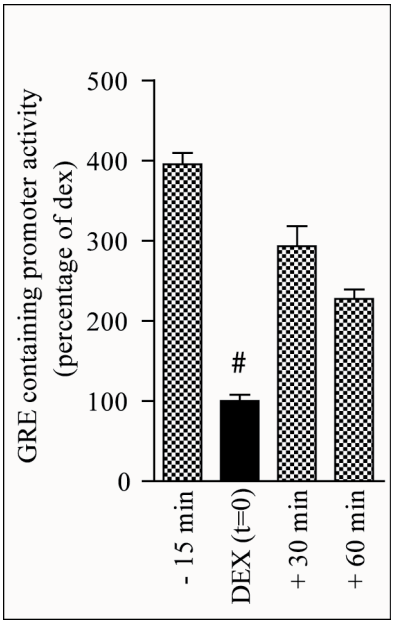

Fig. 3: TAT3-luc (GRE-containing promoter) activity expressed as percentage of maximal induction after 4 hours DEX treatment (filled bar; t=0). All groups (hatched bars) were treated for 4 hours with DEX and only the time of onset of FSK treatment was different. Forskolin treatment strongly enhanced the transcriptional rate of GR at all time points (# indicates significantly different from DEX group with $p < 0.05$). Pre-treatment with FSK resulted in the highest potentiation of the GR transcriptional rate.

The current data demonstrate that time-dependent interactions between GR and cAMP/CREB can occur at the level of the CRH gene, where these factors seem to functionally compete for the same promoter. We suggest that the observed 'primacy' effect for transcription factor action at this promoter is due to the close proximity of the two response elements involved. The spacing of the elements is such that it is likely that both GR and CREB may bind simultaneously (13). Possibly, sterical hindrance at the promoter due to the formation of larger protein complexes is responsible for the importance of timing of stimuli. Alternatively, CREB-mediated chromatin remodeling events that disfavor GR-binding may account for the apparent 'GR resistance'. The latter option is attractive because sterical hindrance neglects the dynamic nature and short residence time of transcription factors on the DNA (20).

While CREB-driven transcription is repressed by glucocorticoids on a composite promoter such as hCRH, it is unaffected on a 5xCRE-containing promoter (21). On the other hand, glucocorticoid signalling is modulated by FSK-induced PKA activation on both the composite

hCRH and the exclusively 3xGRE-containing promoters. Therefore, PKA activation can determine the transcriptional outcome at glucocorticoid target genes, independent of the presence of CREs in the promoter. We postulate that there is no cross-talk between the GR and CREB off the DNA but that PKA activation modulates GR-mediated transcription through phosphorylation of the receptor. Chromatin immunoprecipitation assays on the human CRH promoter would give additional information on the actual binding of these transcription factors to the genomic DNA *in vivo* or in a stably transfected cell-line. These ChIP-assays would allow more molecular insights related to the sequence of binding at the promoter.

It is well known that acute exogenous steroid treatment effectively suppresses stress-induced expression of CRH mRNA in rats (22). However, the current study using a model system shows that repression is markedly attenuated if GR activation is initiated with as little as a 10 minutes delay. Comparable observations were found at the level of ACTH secretion using an *in vivo* perfusion system. Glucocorticoid inhibition of cAMP stimulated ACTH secretion from rat pituitary tissue was shown to be impaired by cAMP treatment prior to glucocorticoid treatment. Both systems show that cAMP activation prior to glucocorticoid treatment results in an impaired action of glucocorticoids at the level of the pituitary and the hypothalamus.

The critical time-window for effective repression by glucocorticoids may have interesting implications in the control of CRH expression *in vivo*. The order of activation of both signalling pathways is variable, and depends on the history of stress and glucocorticoid exposure, as well as the circadian and ultradian pulsatility of glucocorticoid levels (23;24). Therefore, it is likely that effective GR-mediated repression of the stress-induced CRH mRNA expression will only occur in specific situations. We conclude that the differences in timing of stimulatory and repression signals are of consequence for adaptation of the organism to stress.

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Chapter V

Chromatin immunoprecipitation scanning identifies glucocorticoid receptor binding regions in the proximal promoter of a ubiquitously expressed glucocorticoid target gene in brain

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Abtract

While the actions of glucocorticoids on brain functions have been comprehensively studied, the underlying genomic mechanisms are poorly understood. Here we show that GILZ mRNA is strongly and ubiquitously induced in rat brain. To decipher the molecular mechanisms underlying these genomic effects, it is of interest to identify the regulatory sites in the promoter region. Alignment of the rat GILZ promoter with the well-characterized human promoter resulted in poor sequence homology. Consequently, we analyzed the rat 5'flanking sequence by Matrix REDUCE and identified two high-affinity glucocorticoid response elements (GRE) located 2kb upstream of the transcription start site. These findings were corroborated using the glucocorticoid receptor (GR) expressing Ns-1 PC12 rat cell-line. In these cells, dexamethasone treatment leads to a progressive increase of GILZ mRNA expression levels via a GR-dependent mechanism. Subsequently, using chromatin immunoprecipitation assays we show that the two high-affinity GREs are located within the GR-binding regions. Lastly, we demonstrate using multiple tissue in situ hybridisation a marked increase in mRNA expression levels in spleen, thymus, heart, lung, liver, muscle, testis, kidney, colon, ileum, as well as in brain and conclude that the GILZ gene can be used to study glucocorticoid effects in many additional rodent tissues.

1. Introduction

Glucocorticoid hormones modulate brain functioning and are considered essential for adaptation to stress. These hormones exert their actions in the mammalian brain by binding to intracellular glucocorticoid and mineralocorticoid receptors (GR and MR) in target cells (1;2). Upon hormone binding, the activated receptor translocates from the cytoplasm to the nucleus to modulate expression of target genes. In one important mode of action, the ligand-activated receptor scans the genome and interacts with specific response elements in the promoter region of target genes (3-5), termed glucocorticoid response elements (GREs). Once DNA-bound, the receptor coordinates the assembly of coregulator proteins and interacts with the basal transcription machinery to modulate gene expression. The type and concentration of the ligand, the architecture of the GREs and coregulator proteins present in the cell are all known to affect the transcriptional effects of the GR on target genes (6-8). Even within a single cell type GR can either activate or repress the expression of various target genes in this manner (9).

The GR is ubiquitously expressed in brain tissue and is known to be critically involved in the regulation of physiological processes such as neuronal excitability and cognitive functions (10;11). Despite the large number of glucocorticoid target genes identified in different cell lines and tissues, little is known about the physical association of the GR proteins within specific promoter regions in the brain. Previous studies comprehensively described binding of the GR to genome fragments containing GREs in cell lines, as well as in liver (12;13). Hitherto, the mechanisms underlying the genomic actions of glucocorticoids in brain are poorly understood. Therefore, it is of great interest to find a methodology to monitor GR chromatin occupancy in brain tissue. A recently developed method that examine protein-DNA interactions within the context of living cells, *i.e* chromatin immunoprecipitation (14;15), has not yet been performed to study DNA-binding by GR in brain. This approach would allow comprehensive analysis of the dynamics of binding of steroid receptors to the DNA, as well as studying the involvement of coregulator molecules as mediators of glucocorticoid effects in the brain (16).

Large-scale gene expression profiling of brain structures revealed to be challenging due to the high heterogeneity of the tissue samples and because gene expression is highly contextual (17;18). A large number of genes are expressed in brain and relatively small changes in expression levels have been reported after corticosterone treatment (19;20). Therefore, in order to further unravel the genomic actions of glucocorticoids in brain it is essential to find a robustly inducible glucocorticoid target gene that is expressed in rat brain.

In the present study, we sought for a ubiquitously expressed glucocorticoid target gene in brain. We show that the glucocorticoid-induced leucine zipper (GILZ) mRNA expression levels are strongly induced by corticosterone treatment in young adult rat brains. Due to poor sequence homology between the rat and human promoter, we screened the rat sequence for putative GREs using a position weight matrix. In addition, we found that GILZ mRNA expression levels are induced by glucocorticoids in rat Neuroscreen PC-12 cells (Ns-1 PC12) over time in a GR-dependent mechanism. Finally, using ChIP-assays we scanned the proximal promoter of the rat GILZ gene for GR binding, and provide evidence that the two putative GREs are localized within the GR-binding regions. We conclude that GILZ is induced via GR-binding to the regulatory sites in the proximal promoter of the rat gene. Finally, we propose that this promoter and its identified regulatory sites can indeed be used as a new molecular marker to monitor GR chromatin occupancy and transcriptional coregulator recruitment in brain.

2. Material & methods

2.1 Animals and tissue preparation

All animal experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University (UDEC numbers 04052 and 06055). Adult male Sprague-Dawley rats (300g; n=16) were obtained from Harlan (Leiden, The Netherlands) and adult male c57bl/6 mice (26g; n=16) from Janvier (France).

2.2 Multiple tissue in situ hybridisation in c57bl/6 mice

Sixteen adult male c57bl/6 mice were adrenalectomised under isoflurane anaethesia, single housed, and fed with oats containing 400 µg corticosterone or vehicle. Mice were decapitated 3 hours after oats administration, trunk blood was collected and organs were dissected and frozen on dry ice. Two mice were killed at 30 minutes after treatment to determine peak levels of hormone. Plasma corticosterone was determined by radio immuno assay (ICN Biomedicals, Costa Mesa, CA). Peak levels of plasma corticosterone 30 minutes after administration were 28±8 µg/dl. Since there can be a considerable degree of cell-specificity in the transcriptional response to glucocorticoids, we harvested multiple organs and evaluated expression and regulation of the mRNAs using multiple tissue in situ hybridisation. Tissues were cut at 16 µm in a cryostat and collected on poly-L-lysine coated slides for *in situ* hybridisation.

2.3 Regulation and expression of GILZ in brains of Sprague-Dawley rats

To investigate GILZ responsiveness to corticosterone in brain, adrenalectomised male Sprague Dawley rats received a 3mg/kg intraperitoneal (i.p.) corticosterone injection or left untreated (n=8). Blood samples were taken from the tail vein by a small incision with a razorblade every 30 minutes after i.p. injection and collected in EDTA-coated tubes (Sarstedt). Plasma was obtained by centrifugation at 10000 x g rpm for 20 minutes at 4^{o}C and subsequently stored at –20^{o}C until assayed. Three hours after treatment all animals were sacrificed by decapitation. Tissues were cut at 20 µm in a cryostat and collected on poly-L-lysine coated slides. All in situ hybridisation experiments were performed as previously described (21). For RNA isolation and cDNA synthesis, the brains were dissected and the thalamus area was homogenized using the PRO200 homogenizer (Pro Scientific, Oxford , CT, USA). The samples were centrifuged at 13000 rpm 20 min. at 4^{o}C and subsequently stored at –20^{o}C until RNA extraction.

2.4 Oligonucleotides for in situ hybridisation and qPCR

A detailed description of oligonucleotides design for *in situ* hybridisation and qPCR is given in the Supplementary data (material and methods and table 1 & 2 in Supplementary data). Table S1: Sequence of primers designed for mRNA amplification by qPCR of target genes. Table S2: Sequence of primers used to ‘scan’ the proximal promoter of GILZ by by ChIP experiments (rattus norvegicus).

2.5 Tissue culture

Neuroscreen-1 PC12 (Cellomics ,Berkshire, UK) cells were grown and maintained in RPMI 1640, supplemented with 5% Foetal Bovine Serum, 10% Horse Serum, penicillin (20 U/mL) and streptomycin (20 ug/mL) (all Invitrogen Life Technologies, Breda, The Netherlands). To assess whether the rat GILZ gene is induced by GR, a day prior treatment, 1x10^{6} Ns-1 PC12 cells were seeded on collagen-coated (Roche) 100 mm dishes and treated

with ethanol (vehicle) or 10^{-7}M dexamethasone (DEX) for different time points. Next, total RNA was extracted using Trizol reagent (Invitrogen) and cDNA synthesis was performed as aforementioned.

2.6 Chromatin immunoprecipitation assays (ChIP)

Ns-1 PC12 cells were treated for 90 min. with either 10^{-7}M dexamethasone or ethanol. ChIP-assays were performed as described in detail in Supplementary data (figure of ChIP-assays in A549 cells). Fold-enrichment values were calculated as follow: fold enrichment for specific primer = $(GR^{DEX}/Igg^{DEX})/(GR^{EtOH}/Igg^{EtOH})$. Recovery is expressed as percentage of inputs.

2.7 Computational analysis

To identify potential GREs, we processed DNA sequence by Matrix REDUCE in which a position specific affinity representation of the Transfac position weight matrix M00205 (http://www.gene-regulation.com/cgi-bin/pub/databases/transfac/getTF.cgi?AC=m00205) was being used (Batenburg et al., unpublished). This representation is called a position specific affinity matrix, or PSAM (22). In the PSAM every nucleotide in the weight matrix has a value between 0 and 1. The product of the weights evaluated for a short segment associated with a PSAM at a location in the DNA, reflects the likelihood that the element at that locus is a potential GRE. The 3kb human GILZ proximal promoter DNA segment (Ensembl gene ID ENSG00000157514) and its orthologous sequence in rat (Ensembl gene ID ENSRNOG00000013786) were processed to identify potential GREs at various distances upstream TSS measured in nucleotide numbers.

2.8 Statistics

Data were subjected to Student's *t*-test pairwise comparisons. Significance was accepted at $P<0.05$. Results are presented as average ±SD.

3. Results

3.1 Distribution and regulation of a glucocorticoid-induced gene in brain

The aim of present study is to identify a candidate gene for understanding the genomic actions of glucocorticoids in brain. Because for chromatin immunoprecipitation, the amount of tissue is an important factor, we examined GILZ expression and regulation by corticosterone in rat brain. Representative GILZ Hybridisation autoradiographs are shown in Fig. 1. GILZ transcript was ubiquitously detected throughout the brain of adrenalectomised (ADX) animals. The hybridisation signal obtained with mismatch controls did not exceed film background. GILZ mRNA hybridisation signal was the highest in thalamic and hippocampal areas. In hippocampus, the signal was higher in the CA2 and CA3 pyramidal cell layers compared to the CA1. Hybridisation signal was also observed in areas associated with predominant presence of glial cells, such as the stratum radiatum, *i.e.* the border between the CA1 area and the dentate gyrus. In the thalamus, the ventral posteromedial thalamic and the subincertal nuclei showed the highest signal intensity. Cortical areas exhibited moderate homogenous hybridisation signal.

Secondly, to assess whether GILZ is induced by increasing glucocorticoid blood levels, we administered i.p. corticosterone injections to ADX animals. Corticosterone (CORT) plasma concentrations, as measured by radio-immunoassays, revealed to peak at 30 minutes and return to baseline as early as 90 minutes after corticosterone injections (Fig. 2A).

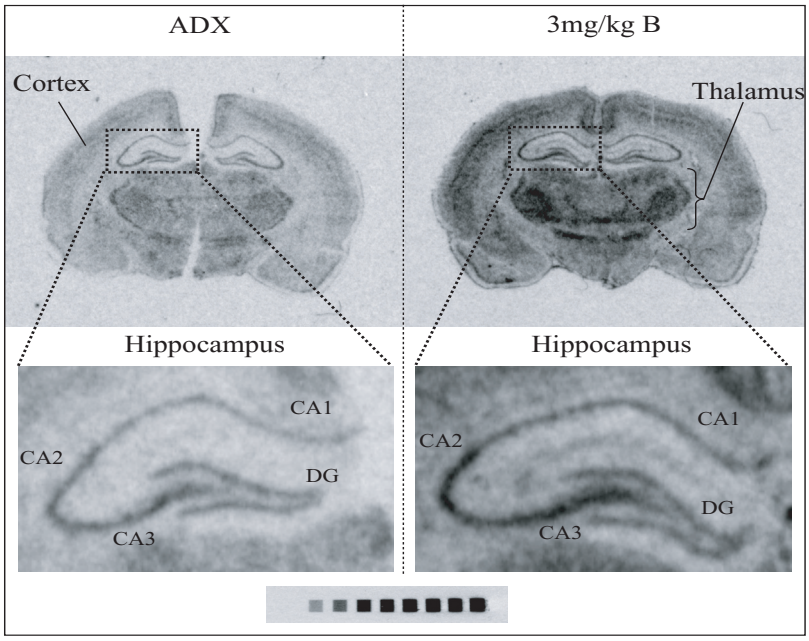

Fig. 1: Neuroanatomical distribution and regulation of GILZ in Sprague-Dawley rats. Representative autoradiographs of GILZ hybridisation signal in untreated and 3mg/kg corticosterone treated ADX animals. GILZ is ubiquitously expressed in rat brain. Following corticosterone treatment GILZ is induced in all hippocampal subfields, cortex and thalamic nuclei. CA: Cornu Ammonis; DG: dentate gyrus; Sub: subincertal nucleus; VPM: ventral posteromedial thalamic nucleus.

GILZ transcript was detected 3 hours after injections (Fig. 1). Overall, GILZ hybridisation signal was higher in all areas studied including thalamus and hippocampus. In hippocampus, treatment did significantly increase GILZ mRNA expression levels in all hippocampal subfields with the highest increase in the pyramidal cell layer of the CA1 area.

To corroborate these findings we tested by means of qPCR the GILZ mRNA contents of thalamic homogenates from ADX animals with and without corticosterone treatment. To control for specificity, the mRNA expression levels of a control gene that is not regulated by glucocorticoids, *i.e.* the potassium voltage-gated channel Kv3.2, were measured in both groups. As shown in Fig. 2B, corticosterone injections in adrenalectomised animals (filled bars) resulted in a 2.4 fold-increase in GILZ mRNA expression levels compared to control animals (open bars). In sum, GILZ is ubiquitously expressed throughout the rat brain and importantly strongly induced by corticosterone treatment. To further study GILZ regulation by glucocorticoids in brain, it is of importance to characterize the regulatory sites in the promoter region of the gene.

3.2 Matrix-based prediction of GREs in rat promoter sequence

Previously, the promoter sequence of the human GILZ gene has been topic of several studies. So far, several functional GREs have been identified in the human promoter (23; 9). A schematic representation of the human GILZ promoter with the precise location of the known response elements is given in Fig. 3A. Alignment of the human and rat promoter sequences resulted in a very low homology; 57% sequence homology in the promoter region

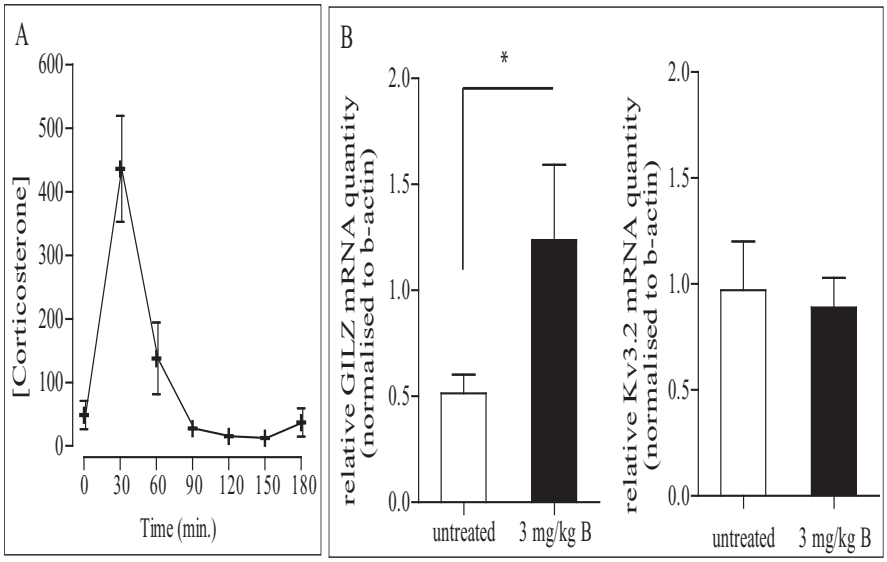

Fig. 2: (A) Plasma corticosterone concentrations at different time points after intraperitoneal injections with 3mg/kg corticosterone in ADX Sprague-Dawley rats. Data are expressed as averages (n=3 ± SD). (B) GILZ and Kv3.2 mRNA levels in thalamic homogenates of untreated (open bars) or corticosterone treated (filled bars) animals. All data were normalized against beta-actin expression levels. Asterisk indicates significantly different from untreated animals.

in contrast to 84% in e.g. exon 3. All previously identified functional GREs in human appeared to be absent or only partially present in the rat sequence. Therefore, to localize putative GREs in the promoter of the rat gene, we screened the genomic sequence using a position-weight matrix (Fig. 3B).First, we validated the matrix by applying it to the human sequence (Fig. 3C). Two GREs were detected; the first GRE located at -1958/-1944 bp (GGAACCcaaTGTTCT) and the second GRE at -2418/-2404 bp (TTAACAgaaTGTTCT) upstream of the transcription start site (TSS). Interestingly, both GREs have an identical second half-site that precisely overlap with the consensus GRE used in the position-weight matrix and known to be essential for DNA binding and transcriptional activity of the GR (24). Using this matrix-based GRE prediction method, we show that within the 3kb sequence upstream TSS of the rat GILZ gene, two putative high-affinity GREs are localized (Fig. 3D). The first one, containing a second half site that precisely overlap with the above mentioned consensus sequence, was found at position -2370/-2355 bp upstream TSS (TCTATActtTGTTCT). The second putative GRE was found at position -1873/-1858 (GGAACCtaaTGTTCC). To substantiate these findings, we further studied the GILZ promoter in the GR-expressing Neuroscreen-1 PC12 (Ns-1 PC12) rat cell-line.

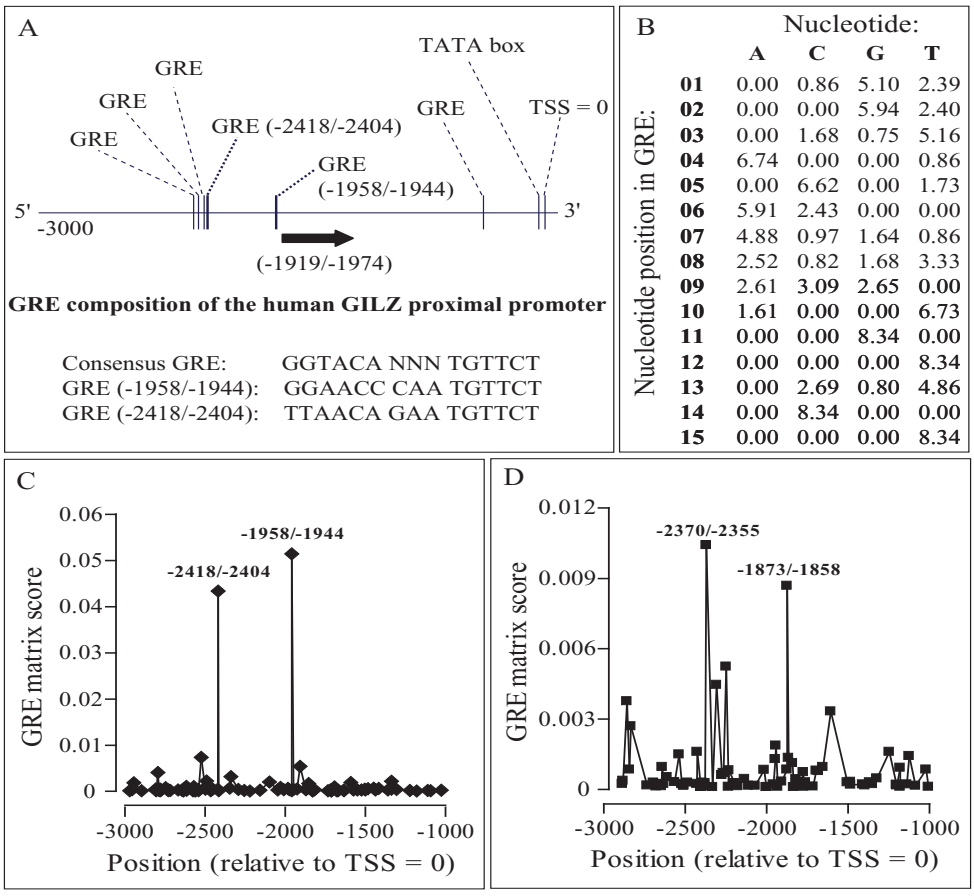

Fig. 3: (A) Schematic representation of the human GILZ promoter and location of the putative GREs relative to the transcription start site (TSS). (B) Position specific affinity matrix (Transfac M00205). Every nucleotide in the weight matrix has a value between 0 and 1. (C) The 3kb human GILZ promoter DNA segment and its orthologous sequence in rat (D) were evaluated using the position specific affinity matrix on potential GREs. The scores represent the products of the weights and reflect the likelihood that the element at that locus is a potential GRE.

3.3 GILZ expression and regulation in rat Ns-1 PC12 cells

In order to gain insight in the molecular interactions between GR and DNA in rat brain, it is necessary to validate the putative GREs in the promoter region of GILZ in a rat cell-line. For this purpose we tested GILZ responsiveness to glucocorticoid treatment in the GR-expressing rat pheochromocytoma Ns-1 PC12 cells. A time course of GILZ mRNA expression levels after dexamethasone (DEX) treatment was performed. As can be seen in Fig. 4, a progressive increase in expression level was found over a period of 6 hours. GILZ mRNA expression levels were significantly induced as early as 2 hours following DEX treatment (4x higher compared to vehicle treated cells), suggesting that promoter occupancy by GR is likely to occur within 2 hours following treatment. Importantly, we have found that GR expression is indispensable for DEX to induce the expression of GILZ mRNA in these cells.

Transfection of a specific shRNA construct directed against the GR (95% knock-down of

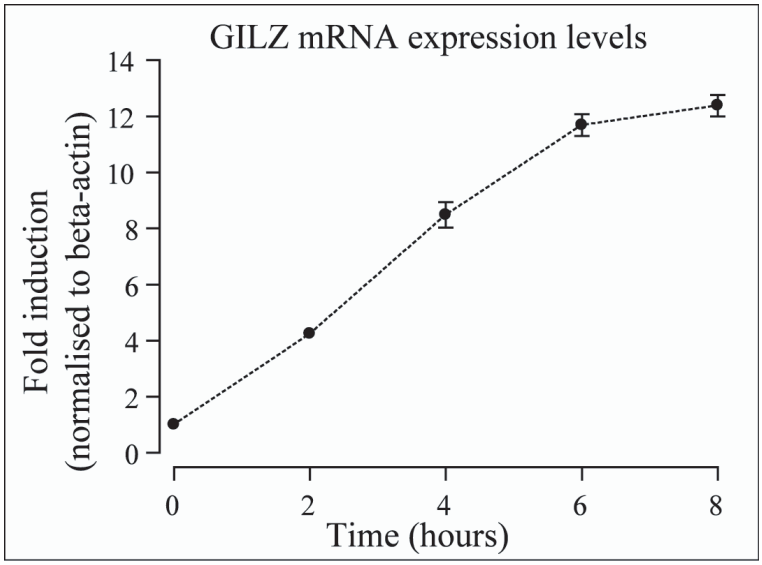

Fig. 4: Time course of GILZ mRNA expression levels in endogenously GR-expressing rat Neuroscreen-1 PC12 (Ns-1 PC12) cells treated with 10-7M DEX. All data were normalized against beta-actin expression levels. GILZ mRNA expression level progressively increases over a period of at least 6 hours of 10-7M DEX treatment. All data are expressed as averages (n=4 ± SD).

the GR protein (8)) resulted in a complete loss of DEX-induced stimulation of GILZ mRNA expression (Dijkmans TF *et al. in preparation*). In sum, DEX treatment induces in a GR-dependent mechanism the expression of GILZ mRNA.

3.4 GR-binding regions in the rat promoter

To identify the specific genomic regions occupied by GR in the rat GILZ promoter, we designed 14 primer pairs for 'scanning' the region up to 5kb upstream of the transcription start site (TSS) of the gene (see table 2 in Supplementary data). All primer pairs were separated by approximately 500bp, *i.e.* the average length of the genomic fragments resulting from sonication. A preliminary scan of the promoter with all 14 primer pairs resulted in a fold-enrichment >3 for primer pairs 6, 7, 8 and 14 using two different GR-specific antibodies (Fig. 5A-B). Signal intensity was the highest around primer pair 7, suggesting the presence of a GRE in this GR binding region. Although both antibodies resulted in a highly similar GR binding profile over the 5kb genomic stretch, the H300 antibody was used for subsequent ChIP experiments because of the higher obtained sensitivity. To validate the preliminary ChIP-scan, we concentrated on GR-binding in the genomic region targeted by primer pair 7. Expectedly, DEX treatment resulted in a significant and marked increase in recovery indicating that this region contains GR-binding elements (Fig. 5C). Importantly, the two high-affinity GREs predicted by computational analysis are located precisely within the GR-binding region identified by ChIP-scan. The region targeted by primer pair 7 is located exactly in between the two predicted GREs, reflected by the highest fold enrichment observed with the ChIP-scan using this primer pair.

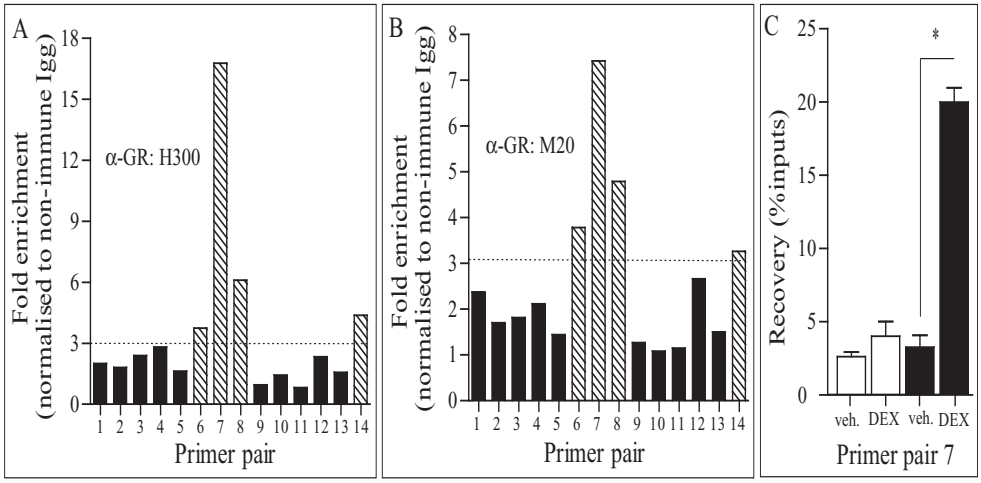

Fig. 5: ChIP-scanning of the promoter region of the rat GILZ gene. (A) H-300 and (B) M20 GR-specific antibodies result in highly similar GR binding profiles in the promoter region of GILZ. The fold enrichment values for the amplified regions were determined by normalizing to the normal rabbit Igg value. The data represent specific enrichment in DEX-treated cells divided ethanol-treated cells. Hatched bars indicate the primer pairs which were enriched >3-fold. (C) Recovery expressed as percentage of the inputs after immunoprecipitation with the H-300 GR-specific antibody and the Igg-control. 10-7M DEX treatment results in a significant higher recovery compared to the ethanol treated cells of the region amplified by primer pair 7. Data are expressed as averages (n=3 ± SD). Asterisk indicates significantly different to ethanol treated group.

3.5 Multiple tissue in situ hybridisation in adult male c57bl/6 mice

In order to assess its use as a marker for GR and MR activity in a more comprehensive manner, we performed GILZ mRNA in situ hybridisation on available multiple mouse tissues harvested 3 hours after a single dose of corticosterone. GILZ mRNA was found to be induced in spleen, thymus, heart, lung, liver, muscle, testis, kidney, colon, ileum and importantly in brain (Fig. 6). GILZ mRNA expression levels were induced by corticosterone in all tissues studied, although differences in the extent of induction were observed. For example, in liver the induction is much higher than observed in testis.

4. Discussion

The glucocorticoid receptor is ubiquitously expressed in brain tissue and is known to be critically involved in the regulation of neuronal excitability and cognitive functions via GRE binding (10). In present study, we sought for a robustly induced glucocorticoid target gene in brain and described the regulatory sites in its promoter region. GILZ was found to be ubiquitously expressed in brain tissue of young adult male rats and strongly induced by corticosterone i.p. injections. Because, the promoter sequence of the gene in rat has not yet been studied, we identified by computational analysis two putative GREs located approximately 2kb upstream of the transcription start site (TSS). Furthermore, using Ns-1 PC12 rat cells we show that GILZ is induced by DEX treatment via a GR-dependent mechanism. Importantly, we provide evidence that the two putative high-affinity GREs identified by computational analysis are located exactly within the GR-binding region of the promoter. We conclude that the transcriptional activity of the rat proximal promoter of GILZ is induced by GR via DNA binding. In sum, we propose that the promoter of the GILZ gene is suitable for studying GR-binding to DNA in specific brain structures using ChIP-assays.

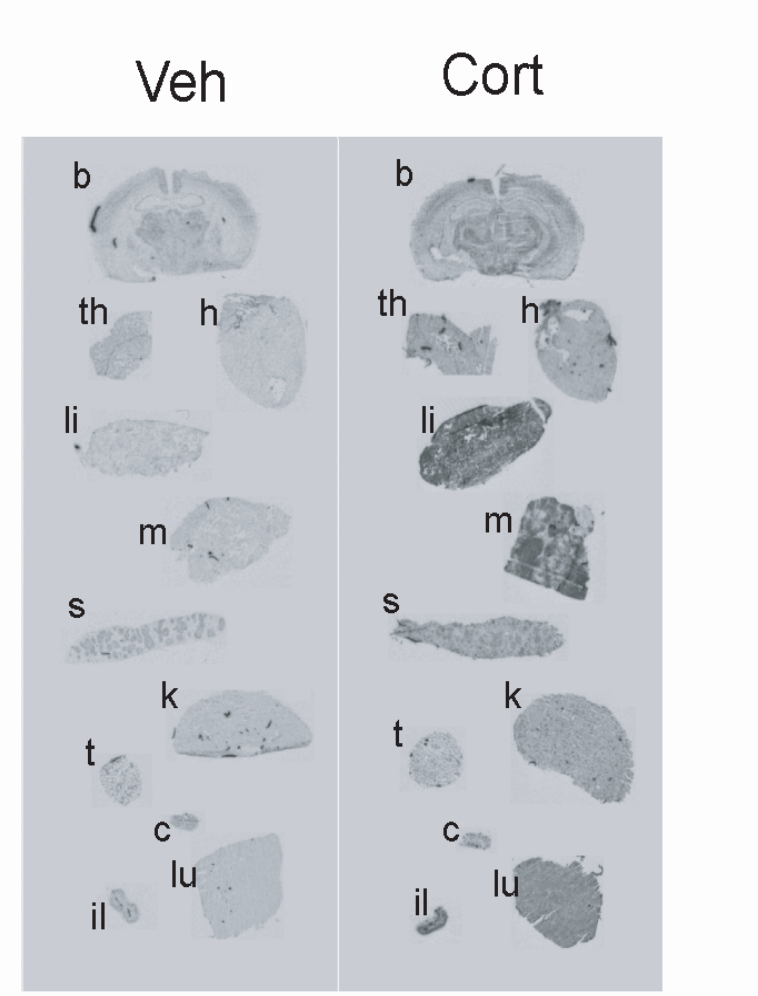

Fig. 6: Distribution and regulation of GILZ in adrenalectomised adult male c57bl/6 mice fed with oats containing 400 µg corticosterone or vehicle. All animals were sacrificed 3 hours after oats administration Legende: b(rain), th(ymus), h(eart), li(ver), m(uscle), s(pleen), k(idney), t(estis), c(olon), il(eum), lu(ng).

A recent study suggests that the precise binding sequences at individual GREs found in responsive genes are strongly conserved throughout evolution (25). Paradoxically, we found that the homology between human and rat proximal promoter sequence was low. The GREs previously described in human differed in nucleotide composition from the GREs identified in present study; additionally, their relative spacing as well as the distance to the transcription start site is somewhat different. Nevertheless, GILZ mRNA expression is induced by glucocorticoid treatment both in human (26) and rat tissue likely through a GR binding region located at approximately 2kb upstream TSS. Specificity of the ChIP-scanning was confirmed by using two different GR-specific antibodies. Immunoprecipitation reactions with both antibodies resulted in highly similar binding profiles showing the highest enrichment surrounding primer pair 7 (Fig 3). A common characteristic of most GRE sequences described

in both species is the presence of the conserved TGTTCT half-site known to be essential for GR-binding to DNA . Additional reporter assays with point-mutations in the GREs would give insights in the relative importance of these GREs. Importantly, in the human A549 cells, GR-binding to DNA was shown to be strongly correlated (nearly 90%) with genes that are glucocorticoid responsive. Taken together, identification of a GR-binding region in the proximal promoter of the rat GILZ gene is sufficient to monitor the action of GR and its associated proteins.

Among the four recently characterized GILZ isoforms, the 137 amino acid long variant with a molecular mass of 17kDa, termed GILZ1, is the originally identified glucocorticoid induced protein in humans (27). The genomic organization of the GILZ gene has only been studied in mouse and human and revealed the presence of two promoter regions. A 5' distant TATA-less promoter containing a CpG island and Sp1 sites, separated by approximately 55kb with the GRE-containing proximal promoter (28). Because no GREs were identified in this TATA-less distant promoter, it is likely that the glucocorticoid-responsiveness of the gene is exclusively mediated by the promoter sequence described in the present study.

Issues that can now be investigated include the relative occupation of GRE containing promoters by mineralocorticoid and glucocorticoid receptors (MR and GR) as a function of circulating steroid levels, and recruitment of coactivator proteins that mediate the effects of steroid receptor activation. GILZ has been shown to be induced by aldosterone via MR in kidney cells (29). In brain, the MR is mainly expressed in limbic structures, such as the hippocampus, and its activity plays an important role in mediating the effects of corticosterone on hippocampal excitability, cognitive function and hypothalamus-pituitary-adrenal axis axis activity (30). Our data show that in the CA3 neurons of the hippocampus, GILZ is induced by corticosterone treatment. Because these neurons express minimal amount of GR and high amount of MR (31;32), we suggest that the CORT-induced mRNA levels in CA3 are possibly meditated by the MR. Therefore, the current assay could readily be adapted to study MR chromatin occupancy in brain.

The neuroanatomical distribution of GILZ mRNA in the current study (in rat) was found to be in part similar to the recently mapped expression in mouse (33). Among the differences observed, the most compelling is found in the hippocampal subfields. We show a marked difference in basal expression in the hippocampal subfields of the Sprague-Dawley rats. The strong hybridisation signal observed in the CA2 and CA3 subfields clearly differed from the much lower signal in the CA1. In the C57BL/6J mice Yachi *et al.* reported a homogenous distribution of GILZ mRNA expression over all hippocampal subfields. Additionally, regulation of GILZ mRNA expression levels by glucocorticoids was also studied in these C57BL/6J mice. A single water-immersion stress significantly increased GILZ mRNA expression in the medial prefrontal cortex (mPFC) and hippocampus in by approximately 2 fold, as measured by qPCR. This effect required the presence of the adrenal glands (33) and therefore glucocorticoids. In line with these results, in Sprague-Dawley rats we measured a 2.4 fold-increase in thalamic homogenates of corticosterone treated animals. The combined results of the experiments in rats and mice suggest that an increase in corticosterone is both necessary and sufficient for the induction of GILZ after stress.

Although the function of GILZ in brain is currently not known, it was originally identified as a dexamethasone-induced gene in murine thymocytes and contains a leucine zipper which is characteristic for DNA-binding transcriptional regulators (27). The expression of GILZ selectively protects T cells from apoptosis induced by anti-CD3 monoclonal antibody treatment. Furthermore, in the context of the immunosuppressive effects of glucocorticoids,

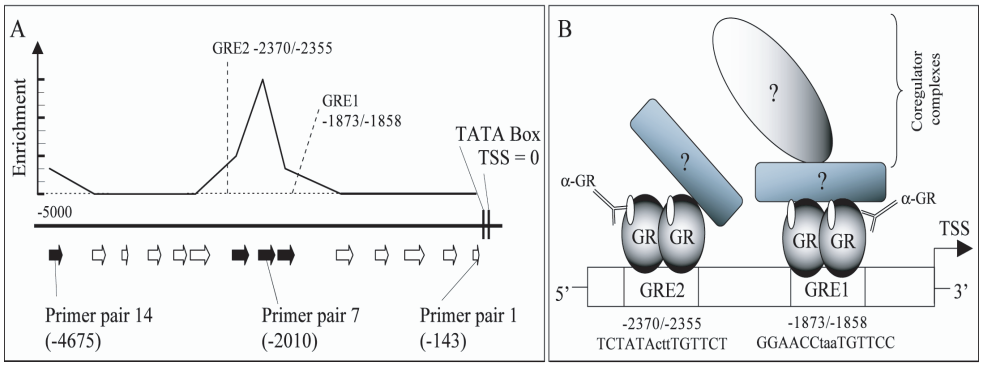

Fig. 7: (A) Schematic representation of the putative GREs and their locus relative to TSS in the rat promoter of the GILZ gene. Primer pair 7 is located exactly in between the two GREs and of all the scanning region was the most enriched. (B) Issues that can be addressed using ChIP-assays on specific brain structures, i.e. hippocampus. The GILZ promoter can be used to study coregulator recruitment by GR on the GILZ promoter in brain.

GILZ was found to inhibit NF- κB nuclear translocation and DNA-binding and to potently suppress AP1-driven transcription (34;35). Through these mechanisms GILZ protein may affect a large number of cellular processes, and regulates NF- κB and AP1-directed gene expression.

Recently it was found that GR-binding invariably takes place at nuclease-accessible sites (36). In addition, the authors showed that these sites are highly cell-specific, implicating that the chromatin organization is a critical determinant for tissue-selective receptor function. We found that GILZ expression and regulation by corticosterone is rather ubiquitous, although the extent of regulation is tissue-specific in adult male c57bl/6 mice (Fig. 6). This likely reflects these cell-specific nuclease-accessible sites and the chromatin landscape. Consequently, we hypothesize that GRE accessibility depends on the chromatin organization in the cell-type and might explain why some GREs were found not to be active in human cells (9).

The type of transcriptional coregulator present in cells is also known to determine the genomic effects of ligand-activated GR (8). Nuclear receptor coregulators are proteins that mediate the transcriptional activity of the GR and may explain cell-specific effects of glucocorticoids action in brain (37). These proteins are enzymatically active proteins that reorganize the chromatin environment and form docking platforms for recruitment of other coregulator proteins (38). We previously described the expression of various transcriptional coregulator proteins in brain, but their recruitment by GR has exclusively been addressed *in vitro* (16;21;39;40). Taken together, the methodological approach described in present study allows the analysis of the coregulator proteins role in the GR-mediated effects within specific brain structures. These experiments are likely to provide valuable information on the mechanism underlying the genomic effects of glucocorticoids (Fig. 7). In this study we have shown that GILZ is transcriptionally induced by corticosterone in many distinct regions in brain of young adult Sprague-Dawley rats. We have also located the GR binding region within the proximal promoter of the gene and identified two putative GREs. Taken together, we conclude that GR and MR chromatin occupancy in specific brain structures can be assessed by ChIP-assays using the GILZ promoter. This approach that examines protein-DNA interactions in intact cells will certainly provide a strong basis to further study essential aspects of glucocorticoid action in brain.

5. Acknowledgments

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6. Supplemetary data

6.1 Oligonucleotide for in situ hybridization

A 45-mer oligonucleotide probe encoding rat GILZ mRNA was 3' end labelled with α [^{35}S] deoxyadenosine triphosphate (GE Healthcare Europe Gmbh, Diegem, Belgium) using deoxynucleotidyl transferase (Promega Benelux, Leiden, The Netherlands). A mismatch control containing 8 evenly spaced mismatches was used as a control for hybridization specificity.

The sequence of the GILZ perfect match oligoprobe is:
TGTTAGGTGTAAAGTTCTCCACATGAGATGACGCTTGGGGAGCCA

and the sequence of the GILZ mismatch oligoprobe is:
GGTTAGGGGTAAATTTCTCAACATGCGATGAAGCTTGTGGAGCCT

6.2 Oligonucleotide primers for qPCR

Primer sequence design was performed by the Primer3 software (Rozen S and Skaletsky H. 2000). Primer pair sequences for GILZ, beta-actin and Kv3.2 mRNA expression are listed in Table S1 (supplementary data).

Gene ID	Forward	reverse
GILZ (rat)	agcgtggtggccctagacaaca	caaccagctcacgaatctgctcctt
Beta-actin (rat)	tgaccgagcgtggctaca	cagcttctctttaatgtcacgca
Kv3.2 (rat)	ctctgtaatttgcagcaaaacca	agcttcaagaaatgcccacaa

Table S1: Sequence of primers designed for mRNA amplification by qPCR of target genes.

Specificity of amplification of each primer pair was controlled by BLAST search against the rat genome. Expression levels of each gene of interest were normalized against beta-actin expression levels in tissue of interest, i.e. rat thalamus or Ns-1 PC12 material. The qPCR oligonucleotide primer pairs for ChIP-scanning were designed to amplify ± 100 bp fragments and were separated by ± 500 bp upstream (5'). Primer pair 1 (pp1) is the first pair upstream transcription start site (TSS = 0 bp). Given the average size of the sheared DNA fragments (±800bp), the 14 primer pairs should effectively scan the promoter for GR occupancy in a 5kb stretch upstream of the TSS. Primer pair sequences for promoter scanning are listed in Table S2 .

Name (primer pair)	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)	Amplified region (primers included)
pp1	aggttcagggaggggatgtggtt	cgtcaggggccatgcaaataagt	73	-143
pp2	gactgaggggttagtcggctgga	aagctctttgggtgccagtcagc	116	-423
pp3	gggagcagtctctgttgccactc	tctggaacccactcaacccatctc	175	-701
pp4	aaagaggggaggaagggctgga	gtgagctaatctcgcagccatcg	94	-969
pp5	ggcatgtccctcttggtgtcgat	tgacacataaactgcgcctcagga	145	-1287
pp6	cctctcgttctgggttggttggt	aggtggcatctgagccttgaggt	164	-1860
pp7	cctcctttgctgataagaggtcccagt	accaaccaacccagaacgagagg	173	-2010
pp8	aatggtctgaaatgggcttatgcaa	ggccttgacctcttcttggcttt	173	-2267
pp9	gcggcaggggcagataatagat	aaggaggagcgagtggtctcgaa	200	-2664
pp10	tcttcccaccatctcccttggaa	tgccgcctagagctttcttggtc	121	-2779
pp11	ggtacagccagcgcaatgtcaac	ggattgcccacaagactgcactg	142	-3085
pp12	ccactgatgtttgctggcatcct	caggtaaggaggaagaacgggtga	62	-3372
pp13	tgccctgaactcacagaacccttc	gcccctactcactggcatcctca	169	-3846
pp14	ggttgcattctcccaacccaaac	gctaggcccttcactggctttca	190	-4675

Table S2: Sequence of primers used to 'scan' the proximal promoter of GILZ by by ChIP experiments (rattus norvegicus).

6.3 Chromatin immunoprecipitation assays (ChIP).

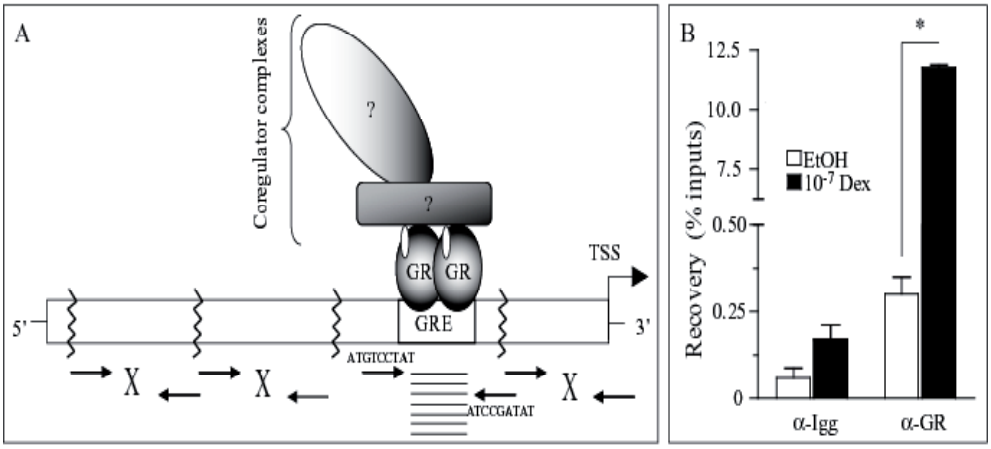

Figure S1: (A) Schematic representation of the principle of chromatin immunoprecipitation assays. (B) Optimization of the ChIP-procedure using the human A549 cells and the previously described GR binding region in the GILZ promoter.. Data are expressed as averages (n=3 ±SD). Asterisk indicates significantly different to ethanol treated group.

Ns-1 PC12 cells were treated for 90 min. with either 10-7M dexamethasone or ethanol. Following treatment, cells were formaldehyde cross-linked (1% formaldehyde concentration) for 10 minutes and incubated with glycine to stop the cross-linking reaction. Cells were rinsed twice, scraped in 2ml 1xPBS with protease inhibitors (Roche), collected in 1.5ml eppendorf tubes and centrifuged at 2000rpm for 5 min. at 4°C. Pellets were rinsed and resuspended in 300µl RIPA lysis buffer (0.1% SDS, 1% DOC, 150mM NaCL, 10mM Tris

pH 8.0, 2mM EDTA, 1mM NaVO3, 1%NP-40, β-glycerolphophate and Na-butyrate). Next, samples were sheared using a Branson Sonifier 250 fitted with a 3mm microtip in 8x10-sec. bursts followed by 1 min. of cooling on ice. The procedure resulted in DNA fragment sizes of 0.3-1.5 kb. Pre-coated 50% slurry Protein A beads (Pierce) in 1×RIPA Buffer containing 20μg/ml sheared salmon sperm DNA and 20mg BSA were added to the pre-cleared samples and incubated with rotation for 60 min. at 4°C. Next, samples were centrifuged at 2000 rpm for 2 minutes and the supernatant was transferred to fresh 1.5 ml eppendorf tubes. Either 6μg of GR-specific H300 (Santa Cruz sc-8992) or M20 (Santa Cruz sc-1004) or normal rabbit Igg (Santa Cruz sc-2027) antibody were added and incubated with rotation overnight at 4°C. Pre-coated Protein A beads (50% slurry) were added and incubated with rotation for 2 hours at 4°C. Beads were centrifuged at 2000 rpm for 2 minutes and washed extensively with following ice-cold buffers: 1x with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl and 20mM Tris-HCl pH 8.0), 4x with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl and 20mM Tris-HCl pH 8.0), 1x with LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% Na DOC, 1mM EDTA and 10mM Tris-HCl pH 8.0) and 2x with TE pH 8.0. Following the last washing step, samples were centrifuged at 2000 rpm for 5 minutes and eluted twice with 250μl elution buffer (1% SDS, 0.1 M NaHCO3) with rotation for 10 min. at room temperature. Samples were reverse cross-linked by incubation at 65°C overnight with proteinase K. DNA was phenol-CHCl3 extracted once and ethanol precipitated in presence of glycogen. Pellets were resuspended in 20 μl TE and assayed by qPCR.

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Chapter VI

General discussion

The main objective of the experiments described in this thesis was to understand the role of coregulator proteins in brain. More specifically we examined their role in the cell-specific actions of glucocorticoids on gene expression in brain. Despite intensive research to decipher the mechanisms by which glucocorticoids mediate their cell-specific effects in brain over the last two decades, the current knowledge does still not satisfactorily explain this phenomenon. One major breakthrough was the discovery of transcriptionally active proteins that neither are receptors nor directly bind to DNA, *i.e.* the coregulator proteins (1-3). It was demonstrated that (cortico)steroid receptor driven transcription is modulated by the type of coregulator expressed in the cells (4;5). The implications of this finding are that 1) the affinity of the steroid receptor for the individual coregulators and 2) the expression levels of these coregulators in a certain cell type are, in addition to ligand availability, critical aspects for determining the genomic effects of glucocorticoids.

The initial observations that SRC1 splice variants are expressed in brain and display striking differences in expression levels at glucocorticoid target areas form the foundation of the thesis (6). First, these findings were substantiated with the mapping, in rodent brain and pituitary, of the two best described corepressors, *i.e.* N-CoR and SMRT (7). Then, based on these neuroanatomical observations, the role of the coactivator and corepressor proteins in GR-mediated transcription was assessed in cultured cells (8). Finally, a method that allows the study of DNA-binding and coregulator recruitment by GR *in vivo* was established. In the current chapter, the expression of coregulator proteins in brain, the co-expression of coregulators with corticosteroid receptors in relevant brain areas and their interactions will be discussed. Furthermore, their role on the expression of the human corticotrophin-releasing hormone (CRH) gene is described. The implications of these findings are discussed in the context of the parvocelullar neurons of the paraventricular nucleus (PVN) of the hypothalamus. Finally, future prospects including new approaches are presented which may lead to further understanding of the role of coregulators in brain.

1. Coregulators and corticosteroid receptors in brain

1.1 Expression of coregulators in brain

The hypothesis that coregulator proteins are involved in mediating cell-specific effects of glucocorticoids is based on the pioneering observation made by Meijer *et al*. almost a decade ago (6). In that study, the expression levels of SRC1 splice variants were found to be largely overlapping but in part highly cell-specific, suggesting different functions of these coactivators. Since then, the expression of various coregulators was mapped in the rodent brain. Recently, a comprehensive exploration of the expression levels of ~20 000 genes, among which many coregulators, in the adult mouse brain was accomplished. All the expression level data of these ~20 000 genes were recollected in one databank from which the Allen Brain Atlas was generated (**www.brain-map.org**) (9). According to the Allen Brain Atlas, a number of these coregulators revealed highly cell-specific expression patterns in brain, corroborating the working hypothesis that these proteins may shape the cell-specific effects of glucocorticoids. Of note, although most coregulators are expressed in many different tissues in the body, to our knowledge only a few coregulators are known to be highly expressed in brain, among which ERAP140 and Nrip-2 (10;11).

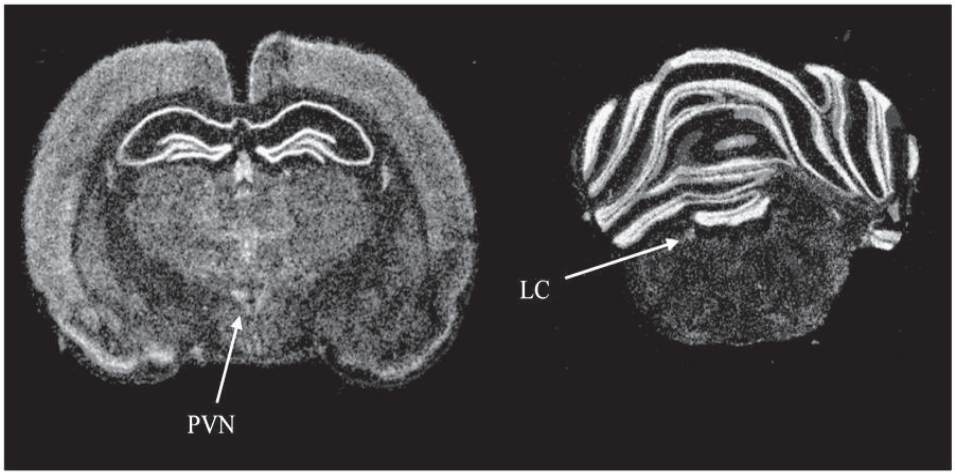
With regard to glucocorticoid signaling in brain, the compelling distribution pattern of SRC1 splice variants should be superimposed on the neuroanatomical distribution of the corticosteroid receptors themselves. The MR has a restricted distribution in brain (mainly in limbic structures), whereas the GR is almost ubiquitously expressed (12). However, the differences in expression levels of SRC1 suggest that both isoforms have specialized effects

on glucocorticoid signaling. In chapter 1, a model was described postulating that 'corepressor and coactivator proteins have opposing effects' on the dose response curve of agonist-bound (cortioco)steroid receptors (13-15). In view of this model it was of interest to explore the expression levels of corepessors in brain. For this reason, the distribution of the first two corepressors identified was determined in rodent brain (in chapter 2). *In situ* hybridization experiments provided proof that both N-CoR and SMRT are expressed in rodent brain and that although their distribution largely overlaps, distinct differences in expression levels were also found.

Presumably, each individual coregulator has a distinct function in MR- or GR-mediated gene transcription. As a result two distinct questions were raised; 1) what is their specific function for the DNA-bound receptor? and 2) how high are their relative expression levels. The first question was approached in chapter 3 and will be discussed at a later stage. For the second, to measure the relative expression level of coregulators within a certain cell-type, a simple graphical methodology was developed (Box 1-see figures).

Box 1

Because of the numerous effects mediated by nuclear receptors in brain, in chapter 2 the cellular distribution of the two best-studied corepressor proteins in the rodent brain and pituitary are described. These corepressor proteins are likely involved in shaping the cell-specific effects of glucocorticoids in brain. To assess the differences in distribution, a readily adaptable method that immediately allows the comparison of the expression levels of two different transcripts can be used. Briefly, the autoradiographs resulting from hybridisation of the N-CoR or SMRT riboprobes on two adjacent sections are scanned. Next, a different color is assigned to both images (red for SMRT and green for N-CoR). Merging the respective images reveals the differences in expression levels between the two transcripts immediately (Figure). Interestingly, when the method is applied on the autoradiographs of N-CoR and SMRT, it is remarkable to find that in HPA relevant regions, marked differences in expression are detected; the locus coeruleus (LC) and hypothalamus are SMRT-enriched areas. This is relevant in view of the catecholaminergic projections originating in the LC that regulate the cellular activity of the CRH-expressing neurons of the PVN. (see colour image page 127).



However, although *in situ* hybridization is the method of choice for the analysis of mRNA expression in brain, it does not provide information on protein stoichiometry. Therefore, dual-immunofluorescence detection of both corepressors proteins was performed and compared to the mRNA hybridization signal (chapter 2). In sum, we observed clear regional differences in brain for the first identified and most extensively studied coregulators, *i.e.* SRC1 isoforms, N-CoR and SMRT.

1.2 Interaction of GR/MR with brain-expressed coregulators

The accumulation of data over the last five years on coactivator expression in brain (11;16;17), and more specifically in glucocorticoid target cells, needs to be substantiated with experiments showing direct interactions between corticosteroid receptor and coactivator. Evidence for direct interaction between the SRC1 isoforms and both corticosteroid receptor types was provided by mammalian 1-hybrid studies (18). In these experiments, expression plasmids encoding fragments of the SRC1 protein fused to the strong activator domain of the herpes simplex virus 16 protein were cotransfected with the MR or GR expression plasmids in a reporter system. As expected, both corticosteroid receptor types were found to interact with the LxxLL-containing fragments (NR-box) of the SRC1 proteins (fig. 1).These LxxLL motifs were previously shown to be necessary and sufficient for interaction between steroid receptors and interacting proteins such as coregulators (19;20). However, a specific interaction between the N-terminal part of the MR with the Q-rich domain of SRC1 (amino acid sequence 988-1240) was also reported. Remarkably, this interaction was specific for the MR and occurred with a fragment lacking a NR-box. This receptor-specific interaction with SRC1 likely results in different receptor-coactivator protein surfaces and therefore will lead to different chromatin remodeling activities and/or recruitment properties of the receptor-coactivator complexes.

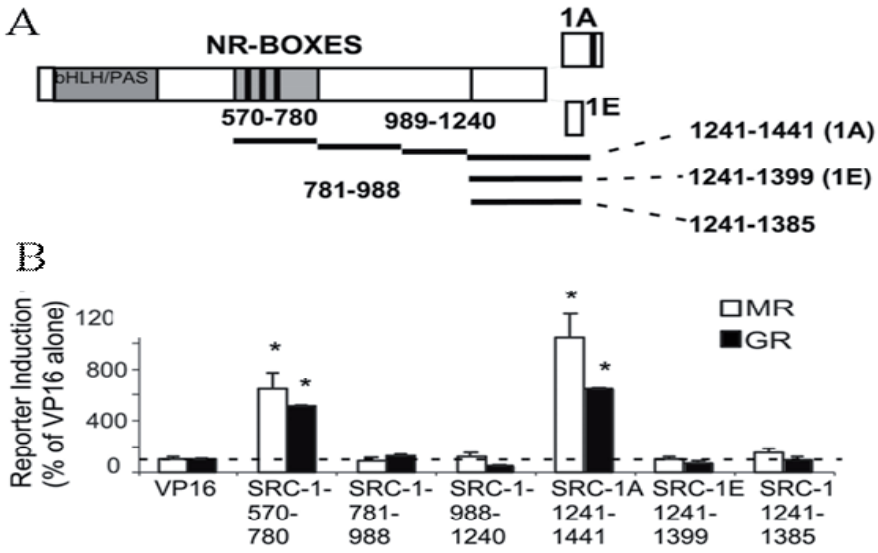


Fig. 1: Protein-protein interactions between fragments of SRC1 and the MR and GR. (A) schematic representation of the SRC1 protein fragments. (B) Reporter activity after co-transfection of the SRC1-VP16 chimeras with MR (open bars) or GR (filled bars). The LxxLL-containing fragments interact with both corticosteroid receptors (18).

1.3 Function of coregulators on transcriptional activity

The observation that SRC1 differentially interacts with the MR and GR suggests that the coactivator causes receptor-specific effects. We previously tested this hypothesis by co-transfection of the SRC1a or SRC1e along with the MR or GR expression plasmids in a reporter system using a minimal synthetic GRE-containing promoter. Indeed, SRC1e co-expression with MR or GR resulted in approximately 10x and 6x higher transcriptional activity, respectively (18). Besides these anticipated receptor-specific effects of the SRC1 isoforms, unexpected promoter-specific effects of SRC1 were also observed. On a multiple GRE-containing promoter SRC1a was unable to stimulate gene transcription by GR, whereas on a single GRE-containing promoter a marked increase in total gene product was observed. On the other hand, SRC1e overexpression resulted in potentiation of the transcriptional activity of the GR on both promoters tested (Fig. 2).

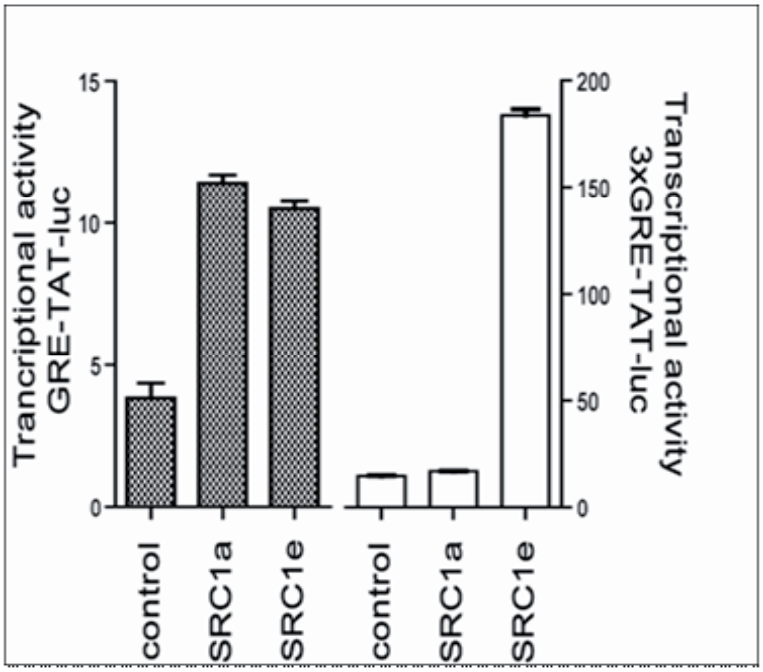


Fig. 2: Promoter-specific effects of steroid receptor coactivator splice variants 1a and 1e on the transcriptional activity of the glucocorticoid receptor. On a single GRE-containing promoter (filled bars) both overexpression of the SRC1 isoforms stimulate GR-driven transcription of the reporter gene. Remarkably, on a multiple GRE-containing promoter (open bars) only SRC1e resulted in potentiation of the transcriptional activity of GR.

These observations suggest differential folding of the receptor complexes upon binding to a single or a multiple GRE containing promoter, which consequently results in different SRC1 recruitment. Another remarkable observation was found when testing the putative GR antagonist RU486 (18). GR-RU486 complexes were found to display different coactivator preferences than corticosterone-activated GR. In sum, SRC1 splice variants-, receptor- and promoter-specific effects that have been observed so far indicate the complex nature of gene regulation by corticosteroid receptors. As on synthetic promoters various levels of regulation were found, the relevance of coregulators for glucocorticoid action in vivo was studied in the more specific setting of the CRH gene.

2. Regulation of CRH expression in brain: the role of coregulator proteins

2.1 CRH-expressing cells in hypothalamus: circuitry and regulation

In brain, CRH transcripts are expressed in distinct regions including hypothalamus, amygdala (fig. 3) and to a lesser extent in hippocampus and neocortex. In hypothalamus, the largest portion of CRH expressing neurons is found in the dorsomedial parvocelullar part of the paraventricular nucleus (21-23). Among the ~2000 CRH expressing neurons in rat, a large part directly projects to the hypophysial portal vasculature, where large CRH peptide-containing vesicles release their contents in the blood vessels. The cellular activity of the parvocellular CRH expressing neurons in the PVN is regulated by catecholaminergic or peptidergic brain stem afferents (including afferents originating in the locus coeruleus), intrahypothalamic (from the arcuate nucleus), hippocampal, amygdaloid and forebrain projections (24).

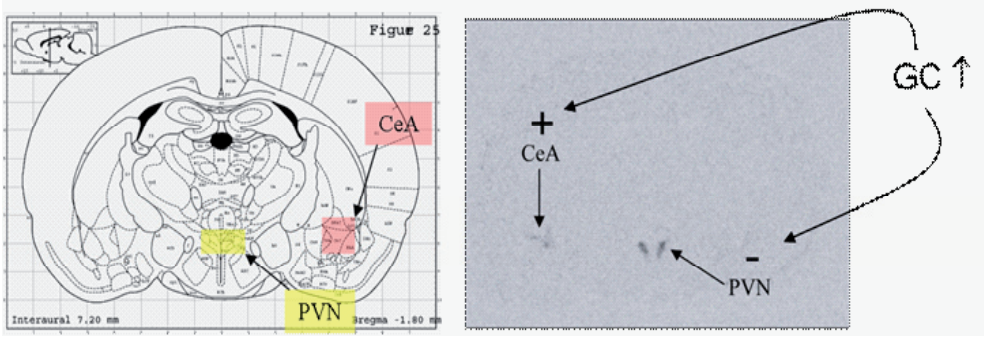


Fig. 3: Increase in glucocorticoid blood levels results in a decrease of CRH expression in the PVN, but concurrently stimulates CRH expression in the CeA in the rodent brain. The mechanism(s) by which glucocorticoids can exert cell-specific opposing effects on CRH gene expression in the rodent brain remains yet unexplained. . PVN = paraventricular nucleus; CRH = corticophin releasing hormone; CeA = central amygdale; GC = glucocorticoids (see coulour image p.122).

Among the various neurotransmitters involved in the regulation of cellular activity of these CRH-expressing neurons, noradrenaline was found to be in a large part involved in the context of stress-induced cellular activation (25). This was supported by the observation that noradrenaline microinjections into the PVN of conscious rats induced a rapid and marked increase in CRH heteronuclearRNA expression (26). Additionally, surgical lesions of the brainstem ascending catecholaminergic projections to the parvocellular part of the PVN significantly decreased the number of CRH-immunoreactive neurons detected after a physical or emotional challenge (27-29).

2.2 Cell-specific effects of glucocorticoids on CRH expression - coregulators

Paradoxically, glucocorticoids repress stress-induced CRH expression in the PVN but stimulate expression in the central nucleus of the amygdala (CeA) (30;31). The aforementioned neuroanatomical distribution of SRC1a and SMRT coincides with these site-specific effects of glucocorticoids. Therefore, to assess the function of these coregulators on GR-mediated regulation of the human proximal CRH-promoter, in chapter 3 we validated a previously described *in vitro* model (32;33). For that purpose, we used the proximal promoter of the human CRH gene known to contain a canonical, functional cAMP response element (CRE) and a negative glucocorticoid receptor response element (nGRE) (fig. 4). Binding of the CRE binding protein (CREB) to the canonical CRE located at the nucleotide position -224 (upstream exon 1) is specifically induced after activation of the PKA pathway with forskolin (34). In addition, deletion of the entire nGRE and specific point mutations results in a loss

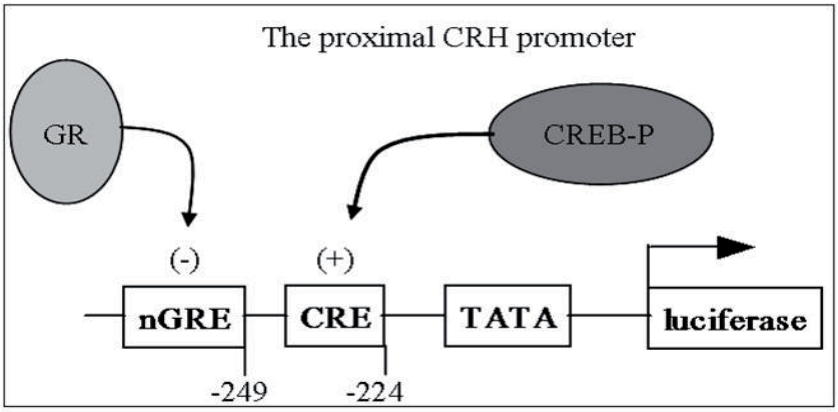


Fig. 4: Schematic overview of the two principal stimulatory or inhibitory inputs of the human proximal CRH promoter. The CRE and the nGRE located at respectively position -224 and -249 (relative to transcription start site = 0) are indispensable for the cAMP and glucocorticoids-induced effects on CRH gene expression.

of repression by the ligand-activated GR, indicating that DNA binding is essential for the glucocorticoid-induced repression (32;33). Conveniently, the AtT-20 cells express GR endogenously and therefore allowed us to test the effects of overexpression of individual coregulators: SRC1 isoforms, N-CoR or SMRT. Our data revealed that SRC1a increases both the efficacy as well as the potency of the GR-mediated repression of the forskolin-induced CRH expression. In addition, SRC1e, which was previously found to be relatively abundant in the CeA, significantly reduced corticosterone-dependent repression. However, the central postulate that coactivators and corepressors mediate opposing effects on GR-driven transcription turned out to be erroneous. Unexpectedly, the corepressors N-CoR and SMRT did not shape the GR-mediated repression but instead inhibited the CREB-mediated stimulation of the gene. We concluded that coregulators mediate context-dependent effects on gene transcription. However, in view of the chromatin modifying properties of the coregulators, further functional studies in stably transfected AtT-20 cells and chromatin immunoprecipitation assays would provide valuable insights in their direct role in the regulation of CRH expression.

In view of the findings on CRH gene regulation by glucocorticoids, an essential issue that remains to be addressed is nuclear colocalisation of GR, N-CoR, SMRT and SRC1 splice variants in CRH-expressing neurons. Immunohistochemical evidence or *in situ* hybridisation data have provided proof of expression of these genes in the PVN, but colocalisation studies have not yet been performed. In addition, expression levels of coregulators have been studied in adult rodent brains but regulation of these genes has not been addressed. Clearly, studies on spatial and temporal aspects of corticosteroid receptor actions would increase the predictive value of the aforementioned findings.

2.3 Crosstalk and timing of stimuli

It is important to bear in mind that production and release of CRH are two different mechanisms. Depending on the cellular context, production and release of CRH are tightly intertwined or partly independent of each other. The production of CRH following the instantaneous release of CRH-vesicles from the terminal buds into the portal vasculature as a response to a stressor must be regarded as an adaptive mechanism of the organism to restore the amount

of CRH in the cells (35). In chapter 3, FSK treatment of the AtT-20 cells, and the ensuing stimulation of the CRH gene, is used as a model for the catecholaminergic activation of the CRH neurons in the PVN following a physical or emotional challenge. Activation of the transmembrane G-protein coupled noradrenergic receptors in CRH expressing neurons, leads to an intracellular increase in cyclicAMP (cAMP) concentration (36), which is mimicked by FSK stimulation. *In vivo*, the order of stimulatory and inhibitory inputs regulating the cellular activity of the CRH-expressing neurons may vary considerably. Therefore, in chapter 4 we tested whether the order of activation of cAMP and glucocorticoid signaling pathways affected CRH gene expression. Interestingly, elevated glucocorticoid levels prior to FSK treatment resulted in a marked reduction of CRH stimulation. On the other hand, mimicking the hierarchical order of cAMP and glucocorticoids cellular regulation after a stressor, *i.e.* first increasing intracellular cAMP concentration followed by GR-activation, resulted in a limited ability of GR to suppress the CRH promoter activity. These findings indicate that circulating glucocorticoid levels and consequently the extent of GR activity prior to a stressful event is a critical factor in modulating CRH gene expression and therefore the CRH response. This finding may be of relevance to understand the interindividual variability to stressors (37). Glucocorticoid blood levels vary over the day with pulses every hour. Recently it was found that a stressor induced a much larger response when triggered during the rising phase of a pulse, than during its descending phase. In sum, the timing of glucocorticoid exposure in relation to that of noradrenergic activation induced by a stressor are of critical importance for the magnitude of the response of CRH expression (38;39).

3. Coregulator proteins: overall *in vivo* and pharmaceutical relevance

3.1 Knock-out animals

Valuable information on the role of coregulator proteins comes from knock-out (KO) animals. These animals allow us to study the gene expression in animals lacking only the gene products of interest, although it was previously shown that in several KO mice compensatory mechanisms are active, complicating the interpretation of the data. However, by observing the expression of several coregulators during development it has become evident that a number of coregulators are specifically implicated in brain maturation (40-42). Partial or total exclusion of various coregulators in knock-out animals resulted in profound effects on neuronal integrity in the adult brain. This is for example the case for the coactivator peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) knock-out animals (43;44). Unpublished data from our lab using SRC1 knock-out (KO) mice indicate the involvement of SRC1 proteins in glucocorticoid actions in brain. Indeed, SRC1 KO and wild type (wt) mice show a difference in CRH mRNA responses after daily injections with dexamethasone during 5 days. Surprisingly, not only glucocorticoid-dependent effects were observed but also CRH stimulation was altered in the SRC1 KO. Altogether, these data suggest that SRC1 is involved in both stimulation and repression of the CRH gene and therefore should be regarded as a central 'regulator' of CRH expression. These findings were corroborated by specific knock-down and overexpression of SRC1 in the AtT-20 cells (fig. 5).

3.2 Coregulator recruitment by GR/MR *in vivo*

Eukaryotic gene control is coordinated by an array of transcription factors and coregulators. Induction of transcription requires the formation of pre-initiation complexes which includes the RNA polymerase II and six $TF_{II}A$ to F complexes (45). Recent studies on the dynamics of transcription factor binding and coregulator recruitment at the core promoter of an endogenous gene, revealed that transcription factors bind periodically with either a fast

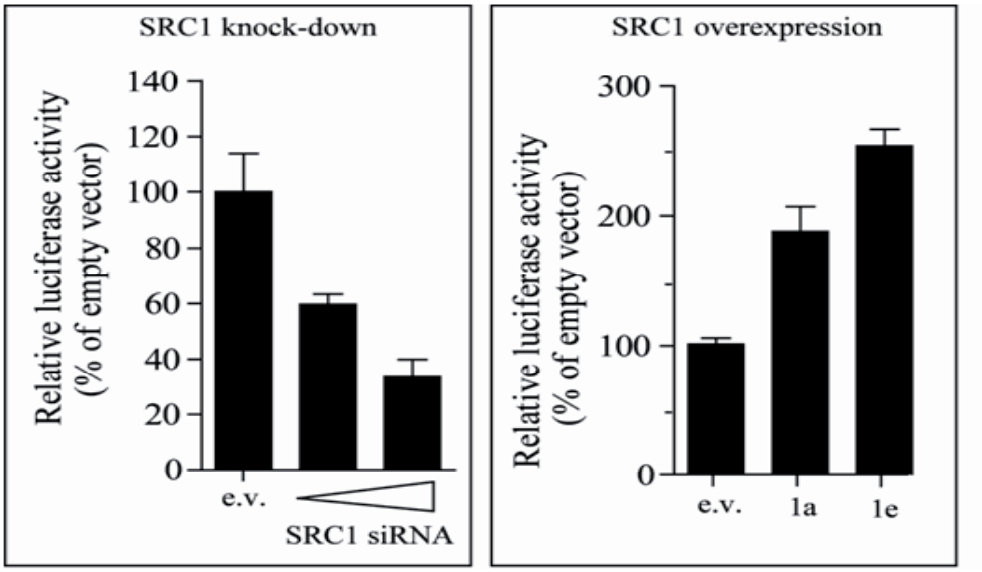


Fig. 5: FSK-induced CRH stimulation in transiently transfected AtT-20 cells. Specific SRC1 knock-down resulted in a loss of FSK-induced stimulation whereas SRC1a or SRC1e overexpression increased FSK-induced CRH gene expression.

(minutes) or a slow cycle (~15 to 90 minutes) (46). The exact functions of the fast and slow cycling of the transcription factor remain uncertain, however it has been speculated that the fast cycling reflects the 'scanning' of the genome and that slow cycling reflects the binding of the transcription factor at the promoter and mRNA synthesis. Recently, chromatin immunoprecipitation (ChIP) based assays on an endogenous regulated promoter, revealed that the first cycle engages reorganization of the chromatin environment without initiating transcription. The following cycle resulted in a stepwise assembly of the transcription initiation complex which is the basis of the formation of active transcription complexes and mRNA synthesis (46-48). Clearly, such studies need to be extended to brain tissue in order to gain insights in the molecular events leading to transcription in specific brain regions.

In chapter 5, a methodological approach is presented that can be used to address such issues. Up to date, several studies describing promoter occupancy of steroid receptors and interacting proteins have been performed *in vitro* but for promoter occupancy *in vivo* and especially in brain still very little is known. Therefore, we tested, based on previous large-scale studies that identified glucocorticoid target genes in several tissues, the responsiveness of the glucocorticoid-induced leucine zipper (GILZ) gene to corticosterone treatment in rodents. We found that GILZ is ubiquitously expressed in brain and regulated by corticosterone treatment. In addition, we identified using an *in vitro* system the GR-binding regions in the rat GILZ promoter and subsequently scanned these segments using a position-weight matrix for GREs. We present a method that should allow studies on the dynamics of transcription factor binding and coregulator recruitment at the core promoter of an endogenous gene in brain. These results should provide valuable information on the mode of action of glucocorticoids in brain.

3.3 Pharmaceutical relevance: dissociating ligands

Glucocorticoids are used clinically as highly effective anti-inflammatory and immunosuppressive compounds, and have been prescribed for more than fifty years for a variety of conditions. Their strong anti-inflammatory and immunosuppressive effects have made synthetic glucocorticoids, such as dexamethasone and prednisolone, among the most successful drugs in history. In spite of their success glucocorticoids have, due to their widespread actions in the body, strong side effects upon prolonged usage. Essential steps of the sequential signal transduction by glucocorticoids are 1) translocation of the receptors, 2) DNA-binding, 3) coregulator recruitment and 4) chromatin reorganization. All mechanisms are at least in part determined by the ligand and the subsequent conformational changes it brings about. Dissociating ligands affect specific aspects of this signal transduction route, and could therefore present interesting compounds because they may dissociate between anti-inflammatory/immunosuppressive properties and side-effects. Many effects of these selective ligands seem to be caused by differential interactions of the GR with coregulators. In fact, such altered interaction between the receptor and its coregulators was also reported for the canonical antagonist/partial agonist for the glucocorticoid receptor, RU486. This antagonist was found to enhance recruitment of N-CoR by the ligand-activated GR (49).

The allosteric changes occurring upon ligand binding govern DNA binding by determining the affinity of the GR for its cognate DNA site. This is best exemplified by the differential promoter occupancy following activation of the GR with a set of fifteen closely related arylpyrazole compounds. In line with the sequential signal transduction of the GR, different ligand-specific histone acetylation profiles were reported for each arylpyrazole compound. Taken together, each ligand constrains the receptor to the regulation of an exclusive set of target genes, caused by ligand-specific allosteric changes (50). An additional example was found for the AL-438 bound GR that has impaired PGC-1 interaction whereas SRC2 recruitment was not affected (51). Likewise, activation of the GR by the nonsteroidal LGD5552 compound resulted in different protein-protein interactions (52).

The aforementioned examples of altered coregulator recruitment by steroid receptors upon binding of various ligands illustrate the advantage several of these ligands may present in the search of safer synthetic glucocorticoids. For example, since SRC1a was found to be involved in the GR-mediated repression of the CRH gene, ligands that impair SRC1a interactions with GR will likely result in altered HPA-axis activity. These findings should contribute to the design of specific ligands that affect only in part glucocorticoid signaling and therefore reduce the unwanted effects that appear upon prolonged usage of glucocorticoids in the clinic.

3.4 Prospectives

One of the major findings described in this thesis is the role of SRC1a in the GR-mediated repression of CRH gene expression. Because, CRH expression is activated in the PVN following a stressor, the *in vivo* relevance of SRC1a can be assessed by studying CRH expression in wild type animals and SRC1 KO mice in the context of an acute stressor. Based on the aforementioned finding, the expectations are that in absence of SRC1a the early glucocorticoid repression of CRH will be impaired in CRH-expressing neurons of the PVN (such as is the situation of SRC1 KO mice). This can be measured by *in situ* hybridization targeted against CRH heteronuclearRNA. To our knowledge this would be the first attempt to study the role of a coregulator protein on gene regulation in a distinct brain area *in vivo*. Additionally, transcriptional coregulators are probably part of the mechanisms involved in the cell-specific effects elicited by nuclear receptors such as the GR and MR in brain. Since up to date ~300 transcriptional coregulators have been identified likely indicating that the

work described in this thesis is probably a starting point to a more extensive research devoted to understanding coregulator function in brain and other target tissue.

3.5 Main conclusions

- The two best-characterized corepressor proteins N-CoR and SMRT are expressed in brain and show distinct distribution patterns suggesting different actions.

- Coactivators and corepessors do not mediate opposing effects on GR mediated CRH gene regulation. The coactivator SRC1a increased the GR-mediated repression of CRH expression and therefore should be considered as a corepressor in this context.

- SRC1 modulates both the stimulatory and repressive signals on the CRH promoter. Overexpression of SRC1 isoforms enhances CREB-mediated transcription while SRC1a overexpression increased both efficacy and potency of the GR-mediated repression.

- GR-mediated repression of CRH gene expression critically depends on the relative timing of GR-activation.

- The glucocorticoid-induced leucine zipper gene (GILZ) is a GR target gene in rodent brain and can be used to study the underlying genomic mechanisms of glucocorticoids in brain as well as in many additional rodent tissues.

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Chapter VII

SUMMARY

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. As a result, blood glucocorticoid levels increase to support return to homeostatic set-point by enhancing e.g. neuroplasticity in the brain which underlies behavioural adaptation.

Currently, the raising awareness of the role of glucocorticoids in the onset of numerous (neuro)-pathologies constitutes the increasing necessity of understanding the mechanisms of action of glucocorticoids in bodily processes and brain functioning. Glucocorticoids mediate their effects by binding to intracellular receptors which act as transcription factors. A remarkable and yet unexplained phenomenon described more than two decades ago, is the cell-specific effects glucocorticoids bring about on gene expression in brain. For example, while glucocorticoids suppress corticotrophin-releasing hormone (CRH) synthesis in the hypothalamus, production of CRH in the central nucleus of the amygdala (CeA) is stimulated by increased hormone levels. Inasmuch as the neuroanatomical distribution of the corticosteroid receptors does not satisfactorily explain these effects, it is of interest to decipher the role of recently discovered coregulator proteins that modulate the direction and the magnitude of steroid receptor-driven transcription. Therefore, in the current thesis the expression and function of central coregulators was studied. In addition, a method that allows detection of coregulator recruitment by steroid receptors in brain tissue was developed.

In chapter 2, the expression levels of two functionally distinct corepressors, *i.e.* N-CoR and SMRT, were mapped in the rodent brain and pituitary. Clear differences in relative mRNA and protein expression levels were discerned in discrete brain areas critically involved in the regulation of the HPA axis such as the PVN. In hippocampus, N-CoR and SMRT were shown to colocalize in the nucleus although complexes containing exclusively N-CoR or SMRT were also detected. Unexpectedly, cytoplasmic N-CoR immunoreactivity was observed in pyramidal neurons of the frontal cortex and of hippocampus, suggesting, an additional role for this corepressor protein, apart form its function in nuclear receptor signalling. These findings strengthen the idea that coregulator proteins may underlie the cell-specific effect of glucocorticoids, particularly if the distinct differences in expression levels of these two functionally distinct corepressors are considered.

In chapter 3, based on the above mentioned uneven distribution in rodent brain of the corepressors and the previously described SRC1 expression levels, the effect of these coregulators on the transcriptional activity of GR was assessed by measuring an endogenous glucocorticoid target gene. In view of the model described in 1999 by Szapary and colleagues coactivators and corepressors were thought to mediate opposing effects on GR-driven transcription. This implies that corepressors were expected to induce a right-shift of the dose-response curve while coactivators should cause a left- shift. Unexpectedly, in the AtT20 cells, a model system for GR-mediated repression of the CRH gene, overexpression of the corepressors did not affect the glucocorticoid-induced repression of the human CRH gene via GR, but rather markedly impaired the cAMP-induced stimulation of the CRH gene. Interestingly, SRC1a overexpression increased both efficacy and potency of the glucocorticoid-induced repression while SRC1e had a tendency to reduce GR-mediated repression. These

observations uncovered the importance of highly abundant SRC1a for CRH gene regulation in the PVN.

Considering that *in vivo* the timing of activation of the two main signaling cascades involved in regulation of CRH expression can vary, in chapter 4 we tested whether this would affect CRH promoter activity. FSK-induction of the CRH promoter was efficiently suppressed by GR when dexamethasone was applied in conjunction to or rapidly after the FSK treatment. On the other hand, a short delay in GR activation resulted in a marked reduction in the ability of GR to suppress the cAMP-stimulated promoter. Since the cAMP and glucocorticoid response elements (CRE and GRE) are situated in close proximity of each other, their sterical hindrance may impair binding of the transcription factors to the promoter. Besides coregulator protein involvement, as described in chapter 3, timing of stimuli in the CRH-expressing neurons of the PVN is likely to be an important parameter for both CRH stimulation and repression.

To further study coregulator recruitment *in vivo*, in chapter 5, we evaluated the glucocorticoid-inudced leucine zipper (GILZ) gene as a potential candidate for chromatin immunoprecipitation (ChIP) assays on brain tissue. GILZ transcript was ubiquitously detected in rat brain and found to be induced after intraperitoneal corticosterone injections. So far, the GILZ promoter and the location of the GREs have been described in the context of human cell-lines. However, alignment of the proximal promoter of the rat and human gene resulted in a low homology (57%). Additionally, the previously identified GREs in the human promoter were not present in the rat sequence. Therefore, we scanned the 5kb proximal promoter of the rat gene by ChIP-assays and identified a GR-binding region. Using a position-weight matrix, we precisely localized two putative GREs in the GR-binding region. Using this method, important issues can be addressed such as coregulator recruitment by GR and MR in specific brain regions.

In conclusion, the findings presented in this thesis extend current knowledge on the neurobiology of stress and may contribute to the design of safer and more selective glucocorticoids with less side-effects. Both biological and pharmaceutical aspects have been discussed in chapter 6 along with future prospectives.

Chapter VIII

SAMENVATTING

Het was de Franse fysioloog Claude Bernard (1813-1878) die het concept van het 'interne milieu' bedacht. Aan de hand daarvan formuleerde de baanbrekende Amerikaanse fysioloog Walter Cannon (1871-1945) aan het begin van de 20ste eeuw het principe van 'homeostase' voor levende organismen. Hij leidde de vakterm als volgt in: 'de gecoördineerde fysiologische reacties die de evenwichtstoestanden in het lichaam behouden zijn zo complex, en zo eigenaardig voor levende organismen, dat er een bepaalde aanduiding voor deze toestanden gebruikt dient te worden- homeostase' (1929). Als homeostase wordt bedreigd, zoals het geval is bij acuut gevaar, wordt er bij de meeste zoogdieren een hormonale kettingreactie vanuit de hersenen geïnitieerd, de zogenaamde hypothalamus hypofyse bijnier as (HPA-as van hypothalamus pituitary adrenal axis in het Engels). Actievering van deze as leidt tot een verhoging van de bloedspiegel glucocorticoïd hormonen die onder andere het terugkeren naar de evenwichtstoestand bevorderen door bijvoorbeeld neuroplasticiteit die ten grondslag ligt aan gedragsmatige aanpassingen in de hersenen te stimuleren.

Het toenemend besef dat glucocorticoïden betrokken zijn bij het ontstaan van talrijke neurologische en andere aandoeningen vormt een sterk argument om verder inzicht te vergaren in de werkingsmechanismen van deze hormonen op lichaamseigen processen (homeostase) en het functioneren van de hersenen. Glucocorticoïden oefenen de meeste van hun effecten uit door binding aan intracellulaire receptoren die op hun beurt genexpressie beïnvloeden (transcriptie factoren). Een opmerkelijk en toch onverklaard fenomeen dat ruim twee decennia geleden beschreven werd, is de celspecifieke effecten van glucocorticoïd hormonen op genexpressie in specifieke gebieden in de hersenen. Terwijl verhoogde hormoonniveaus de synthese van het 'corticotropin-releasing hormone' (CRH) in de hypothalamus onderdrukken, wordt tegelijkertijd de productie van hetzelfde gen CRH in de amygdala juist bevorderd. Aangezien de expressie van de intracellulaire corticosteroïd receptoren in de hersenen al bestudeerd zijn en dat deze de gevonden effecten niet in zijn geheel kunnen verklaren, is het van belang om de rol van onlangs ontdekte 'coregulatoren eiwitten' in dit kader te bestuderen. Deze coregulatoren oefenen invloed uit op de aard en de mate van gen transcriptie. Het onderzoek beschreven in dit proefschrift bestudeert de expressie en de functie van coregulatoren in de hersenen van knaagdieren en in cellijn. Bovendien is er een techniek ontwikkeld die rekrutering (interacties van verschillende eiwitten op het DNA) van coregulatoren eiwitten door de corticosteroïd receptoren in hersenweefsel mogelijk kan maken.

Ten eerste wordt er in hoofdstuk 2 de mate van expressie van de twee eerst ontdekte en functioneel verschillende corepressoren (d.w.z. N-CoR en SMRT) in de hersenen en hypofyse van knaagdieren beschreven. Duidelijke verschillen in relatieve mRNA en eiwit expressie werden aangetoond in hersengebieden die nauw betrokken zijn bij de regulatie van de HPA-as, bijvoorbeeld in de paraventriculaire nucleus van de hypothalamus (PVN: een van de gebieden waar het CRH gemaakt wordt). In de kernen van hippocampale zenuwcellen is er gevonden dat N-CoR en SMRT colocaliseren in dezelfde eiwitcomplexen hoewel ook complexen die uitsluitend N-CoR of SMRT bevatten werden ontdekt. Tegen de verwachtingen in, is bovendien N-CoR immunoreactiviteit in het cytoplasma van piramidale neuronen in de frontale cortex en in enkele hippocampale zenuwcellen waargenomen, hetgeen hoogst waarschijnlijk aanduidt dat N-CoR ook een andere rol uitoefent dan het moduleren van gentranscriptie. De bevindingen beschreven in dit hoofdstuk ondersteunen de gedachte dat deze coregulatoren eiwitten ten grondslag liggen aan de cel specifieke effecten

van glucocorticoïd hormonen in de hersenen. Met name omdat er verschillen in de mate van expressie tussen deze twee functioneel verschillende coregulatoren is gevonden.

Gebaseerd op de bovengenoemde verschillen in expressieniveaus van beide corepressoren in de hersenen en eerder beschreven expressieniveaus van SRC1, worden er in hoofdstuk 3 de effecten van deze coregulatoren beschreven met betrekkeing op de transcriptionele activiteit van de GR op een endogeen responsief gen: CRH. Aan de hand van het model dat in 1999 door Szapary en collega's werd beschreven is verondersteld dat coactivatoren en corepressoren tegenovergestelde effecten zouden veroorzaken op de mate van GR gedreven transcriptie. Dit zou betekenen dat overexpressie van corepressoren een verschuiving van de dosis-response curve naar rechts zou moeten veroorzaken terwijl coactivatoren een verschuiving naar links teweegbrengen. In AtT20 cellen, een bewezen modelsysteem voor het bestuderen van GR-afhankelijke onderdrukking van het menselijke CRH gen, veroorzaakte corepressoren overexpressie geen verschil in de mate van GR-afhankelijke repressie van het gen, maar, resulteerde wel in een vermindering van de cAMP geïnduceerde stimulatie van het gen. SRC1a overexpressie verhoogde zowel de effectiviteit als de potentie van de glucocorticoïd geïnduceerde repressie, terwijl SRC1e een tendens vertoonde om de repressie juist te verminderen. De resultaten benadrukken het belang van de aanwezigheid van SRC1a voor CRH gen regulatie in de PVN.

Omdat *in vivo* de timing van activering van de twee voornaamste signalen betrokken bij het reguleren van het CRH gen sterk kunnen verschillen, is er in hoofdstuk 4 gekeken naar de invloed van de volgorde van activering van de signalen op de activiteit van de CRH promotor. Inductie van de promotor van het CRH gen via de stof forskoline (FSK) wordt op efficiënte wijze door de GR onderdrukt in het geval dat het synthetische glucocorticoïd dexamethason tegelijkertijd of snel na FSK behandeling wordt aangebracht. Echter, een kleine vertraging in het tijdstip van het aanbrengen van dexamethasone had als gevolg een sterk verminderde onderdrukking door de GR van de cAMP gestimuleerde promotor. Omdat de cAMP en GR response elementen (CRE en GRE) in de promotor dicht bij elkaar zijn is er gesuggereerd dat dit effect veroorzaakt wordt door sterische hinder, i.e. dat delen van moleculen in de weg zitten voor andere delen of voor elkaar. Ter zijde van de hoeveelheid en het type coregulatoren dat aanwezig is in de nabijheid van de CRH promotor (zie hoofdstuk 3), is de timing van de activering van de twee signalen ook een belangrijke factor voor zowel stimulatie als repressie van het CRH gen in de PVN.

Om interacties en rekrutering van coregulatoren op het DNA *in vivo* te kunnen bestuderen, wordt er in hoofdstuk 5 onderzocht of het glucocorticoid-induced leucine zipper (GILZ) gen een mogelijke kandidaat-gen is voor chromatine immunoprecipitatie experimenten (ChIP) op hersenweefsel. In vrijwel alle cellen in de hersenen werd GILZ mRNA gemeten en toediening van corticosteron aan ratten veroorzaakte een significante verhoging van de mRNA expressie niveaus. Tot dusver zijn de GILZ promotor en de locatie van de GREs exclusief bestudeert in de context van humane cellijnen. Vergelijking van de nucleotide sequentie (opbouw van het DNA) van de humane en rat promotor toonde aan dat er relatief weinig overeenkomst is tussen beide sequenties (57%). Belangrijker nog, de tot dusver ontdekte GREs in de humane proximale promotor bleken niet of alleen deels aanwezig te zijn in de rat sequentie. Door middel van ChIP-scanning experimenten is er gezocht naar GR bindende gebieden binnen de 5kb opwaarts van de transcriptie start site. De gevonden gebieden zijn vervolgens

onderzocht door de 5kb sequentie te doorzoeken met met behulp van een ‘position-weight’ rooster (bioinformatica). Op deze wijze zijn twee waarschijnlijke GREs ontdekt in exact de promotor gebieden waaraan GR bindt. Door deze techniek te gebruiken kunnen belangrijke aspecten zoals interacties tussen coregulatoren en de corticosteroïd receptoren (GR en MR) eiwitten op het DNA in specifieke hersengebieden worden bestudeerd.

De resultaten beschreven in dit proefschrift dragen bij aan de huidige kennis over de neurobiologie van de ‘stress respons’ en kunnen mogelijk een bijdrage leveren aan het ontwerpen van veiliger en selectieve glucocorticoïden met minder bijwerkingen. Zowel de biologische als de farmaceutische aspecten van de resultaten worden in hoofdstuk 6 uitgebreid besproken.

Publications

Full papers

1. **van der Laan S**, Sarabdjitsingh RA, Van Batenburg MF,Lachize SB, Li H, Dijkmans TF, Vreugdenhil E, de Kloet ER, Meijer OC. Chromatin immunoprecipitation scanning identifies glucocorticoid receptor binding regions in the proximal promoter of a ubiquitously expressed glucocorticoid target gene in brain. J Neurochem. 2008 Aug 14. [Epub ahead of print]

2. **van der Laan S**, Meijer OC. Pharmacology of glucocorticoids: beyond receptors. Eur J Pharmacol. 2008 May 13;585(2-3):483-91.

3. **van der Laan S**, Lachize SB, Vreugdenhil E, de Kloet ER, Meijer OC. Nuclear receptor coregulators differentially modulate induction and glucocorticoid receptor-mediated repression of the corticotropin-releasing hormone gene. Endocrinology. 2008 Feb;149(2):725-32.

4. Meijer OC, **van der Laan S**, Lachize S, Steenbergen PJ, de Kloet ER. Steroid receptor coregulator diversity: what can it mean for the stressed brain? Neuroscience. 2006;138(3):891-9.

5. **van der Laan S**, Lachize SB, Schouten TG, Vreugdenhil E, de Kloet ER, Meijer OC. Neuroanatomical distribution and colocalisation of nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRT) in rat brain. Brain Res. 2005 Oct 19;1059(2):113-21.

6. Ingram N, Martin S, Wang JH, **van der Laan S**, Loiacono R, van den Buuse M. Interaction of corticosterone and nicotine in regulation of prepulse inhibition in mice. Neuropharmacology. 2005 Jan;48(1):80-92.

7. Meijer OC, Kalkhoven E, **van der Laan S**, Steenbergen PJ, Houtman SH, Dijkmans TF, Pearce D, de Kloet ER. Steroid receptor coactivator-1 splice variants differentially affect corticosteroid receptor signaling. Endocrinology. 2005 Mar;146(3):1438-48.

8. Datson NA, Meijer L, Steenbergen PJ, Morsink MC, **van der Laan S**, Meijer OC, de Kloet ER. Expression profiling in laser-microdissected hippocampal subregions in rat brain reveals large subregion-specific differences in expression. Eur J Neurosci. 2004 Nov;20(10):2541-54.

Poster presentations

1. **van der Laan S**, Sarabdjitsingh RA, Lachize SB, Schouten TG, de Kloet ER and Meijer OC. The Glucocorticoid-Induced Leucine Zipper gene: a good candidate gene for chromatin immunoprecipitation on brain tissue.

2. **van der Laan S***,* Lachize S, Schouten TG, de Kloet ER, and Meijer OC. Nuclear Receptor Corepressor and Silencing Mediator of Retinoid and Thyroid hormone receptor mRNA and protein mapping in brain reveals differential expression in glucocorticoid target cells.

3. **van der Laan S**, Meijer OC. Neuroanatomical distribution and colocalisation of nuclear receptor corepressor and silencing mediator of retinoic and thyroid receptors in rat brain.

4. **van der Laan S**, Lachize S, Steenbergen PJ, de Kloet ER and Meijer OC. Function of coregulators in the glucocorticoid receptor mediated regulation of the HPA axis activity: colocalisation studies.

Curriculum vitae

Siem van der Laan, geboren op 10 augustus 1978 te Lusaka (Zambia).

1 december 2008	Postdoc onderzoeker bij de afdeling 'Surveillance et Stabilité du Génome' aan het Institut de Genetique Humaine CNRS UPR 1142 Montpellier, France.
2003-2008	Promotie onderzoek bij de afdeling Medische Farmacologie, aan het LACDR, Leiden, The Netherlands.
1997-2003	Masters opleiding Bio-Farmaceutische Wetenschappen (BFW), Universiteit Leiden, The Netherlands.
2002-2003	Onderzoekstage bij de afdeling 'Behavioural Neuroscience Laboratory' aan het Mental Health Research Institute in Melbourne, Australia.
2001-2002	Onderzoekstage bij de afdeling Medische Farmacologie, aan het LACDR, Leiden, The Netherlands.
1997	Baccalaureat Scientifique (*specialite mathematique*) in Grenoble, France. College du Leman, Versoix, Switzerland.

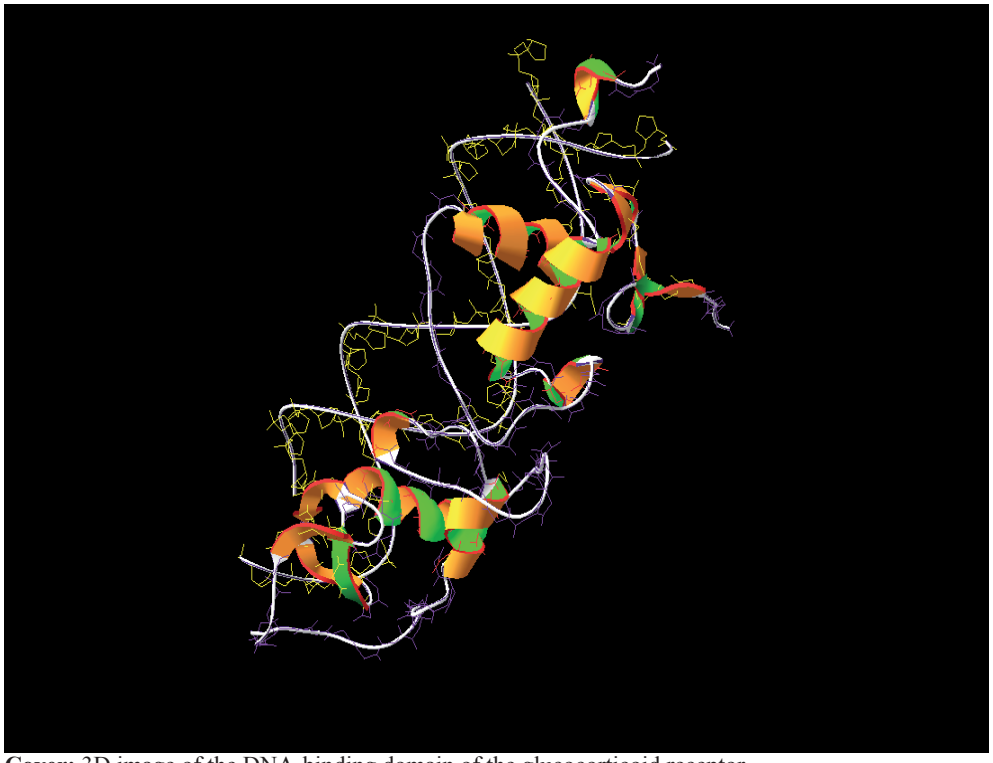
Colour images

Cover image

Cover: Drawing made on 17-09-2008 by Jonas van der Laan on the theme of "Expression and Function of Nuclear Receptor Coregulators in Brain: Understanding the Cell-Specific Effects of Glucocorticoids.

Cover image

Cover: The Glucocorticoid Receptor.
Drawing made in march 2004 by Selwyn van der Laan.



Cover: 3D image of the DNA-binding domain of the glucocorticoid receptor.
reference: Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis 18, 2714-2723.
(url: http://www.expasy.org/spdbv/)

Figure 2, page 11.

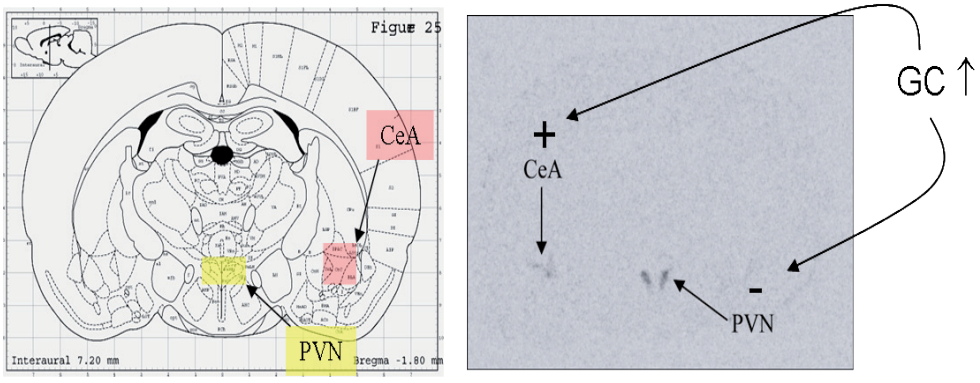


Fig. 2: Increase in glucocorticoid blood levels results in a decrease of CRH expression in the PVN, but concurrently stimulates CRH expression in the CeA in the rodent brain. The mechanism(s) by which glucocorticoids can exert cell-specific opposing effects on CRH gene expression in the rodent brain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

Figure 3, page 35.

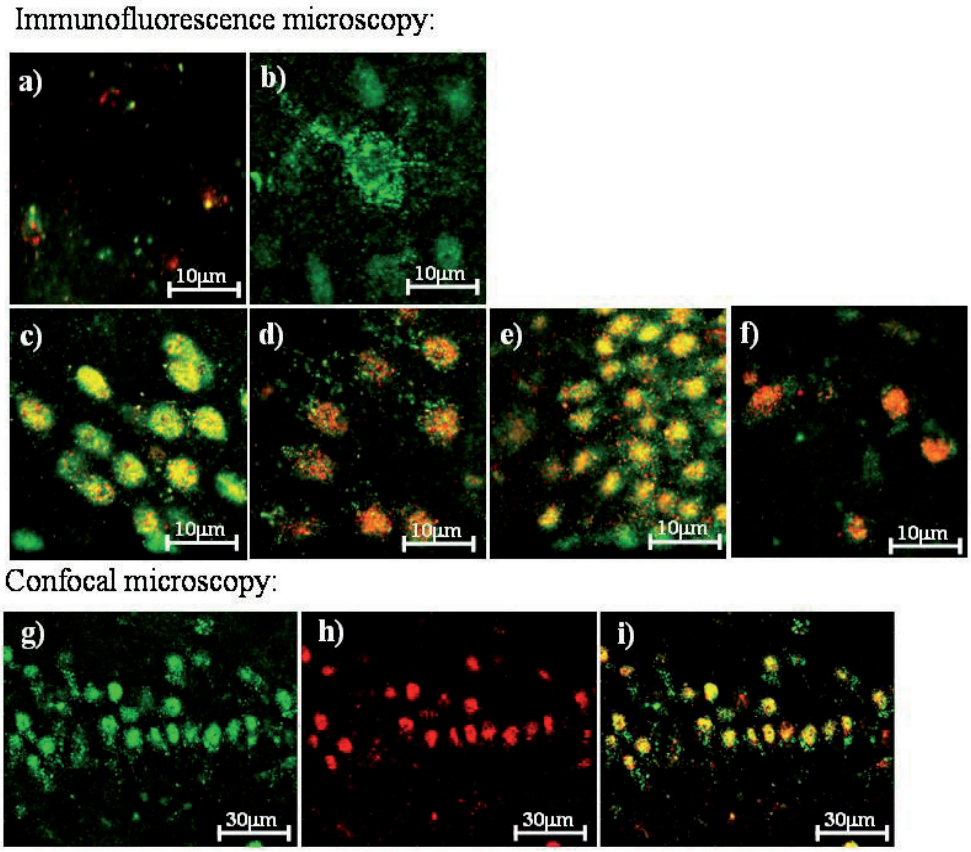


Fig. 3 Dual-immunofluorescence images for N-CoR and SMRT. The merged images show N-CoR (FITC:green) and SMRT (Cy3:red). A: Control IgG image of the aspecific immunoreactivity of non-immune sera. B: Cytoplasmic expression of N-CoR is observed in the pyramidal neurons of the frontal cortex. C-F: Relative expression of N-CoR and SMRT differs in the piriform cortex (c), CA3 (d), dentate gyrus (e) and the locus coeruleus (f). G-I: Confocal microscopy image of N-CoR (g) and SMRT (h) in the nucleus of the CA1 neurons. Colocalisation of the corepressors is shown in yellow (i).

Figure 2, page 66.

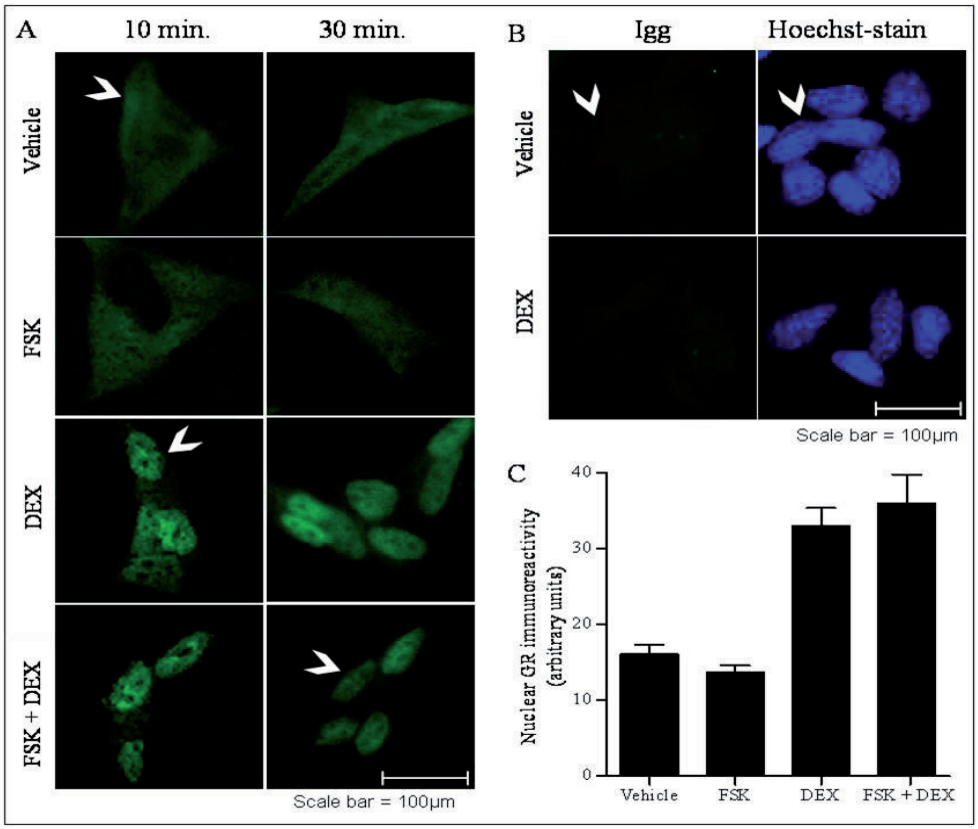
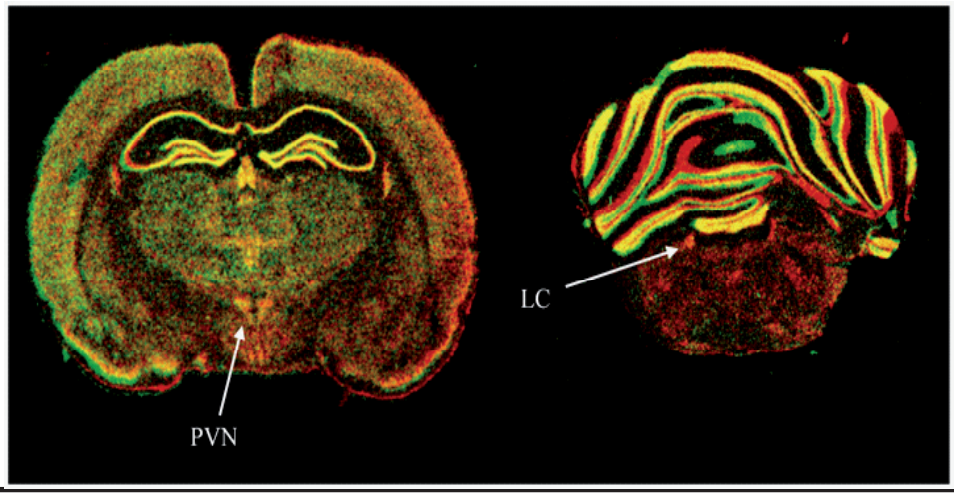


Fig. 2: Immunofluorescent staining of the GR in AtT-20 cells. (2A) Time course of GR-ir in different treatment groups. DEX alone and FSK + DEX cotreatment, show nuclear GR staining after 10 minutes treatment. (2B) Control IgG staining show specificity of the GR-specific antibody. (2C) Nuclear quantification of GR-ir after 10 minutes treatment (The average values ± SEM are shown).

Box 1, page 93.

Box 1

Because of the numerous effects mediated by nuclear receptors in brain, in chapter 2 the cellular distribution of the two best-studied corepressor proteins in the rodent brain and pituitary are described. These corepressor proteins are likely involved in shaping the cell-specific effects of glucocorticoids in brain. To assess the differences in distribution, a readily adaptable method that immediately allows the comparison of the expression levels of two different transcripts can be used. Briefly, the autoradiographs resulting from hybridisation of the N-CoR or SMRT riboprobes on two adjacent sections are scanned. Next, a different color is assigned to both images (red for SMRT and green for N-CoR). Merging the respective images reveals the differences in expression levels between the two transcripts immediately (Figure). Interestingly, when the method is applied on the autoradiographs of N-CoR and SMRT, it is remarkable to find that in HPA relevant regions, marked differences in expression are detected; the locus coeruleus (LC) and hypothalamus are SMRT-enriched areas. This is relevant in view of the catecholaminergic projections originating in the LC that regulate the cellular activity of the CRH-expressing neurons of the PVN.



Nawoord

Hoewel ik met veel passie en plezier onderzoek heb verricht, is de mooiste ontdekking voortvloeiend uit mijn promotie onderzoek dat mijn familie van groot belang is gebleken voor de totstandkoming van dit proefschrift. De liefde van en voor mijn vrouw Jolanda en mijn kinderen Selwyn en Jonas zijn voor mij duidelijk de belangrijkste drijfveer geweest.

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