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

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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).

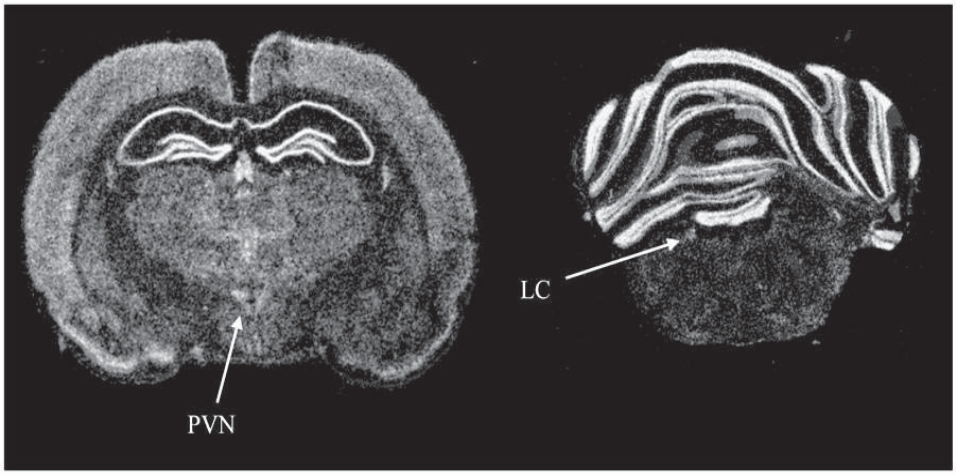
With regard to glucocorticoid signaling in brain, the compelling distribution pattern of SRC1 splice variants should be superimposed on the neuroanatomical distribution of the corticosteroid receptors themselves. The MR has a restricted distribution in brain (mainly in limbic structures), whereas the 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.

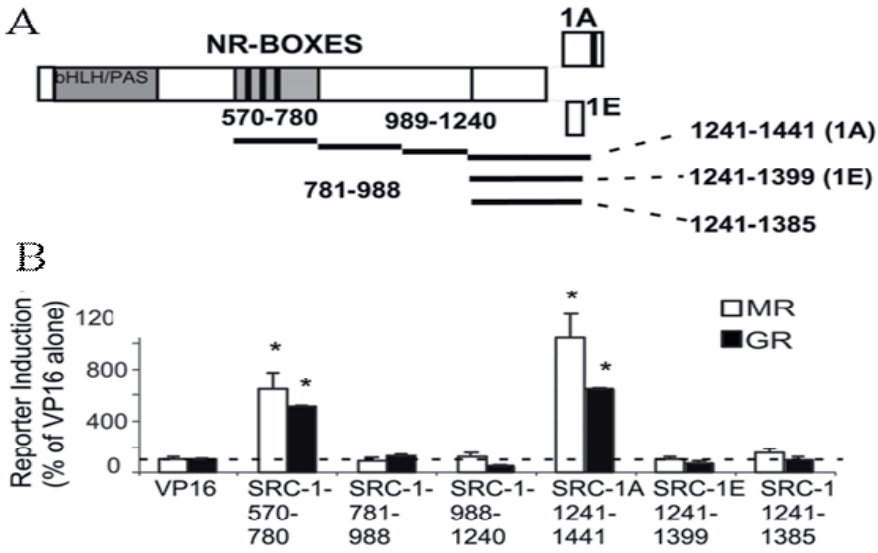


Fig. 1: Protein-protein interactions between fragments of SRC1 and the MR and GR. (A) schematic representation of the SRC1 protein fragments. (B) Reporter activity after co-transfection of the SRC1-VP16 chimeras with MR (open bars) or GR (filled bars). The LxxLL-containing fragments interact with both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

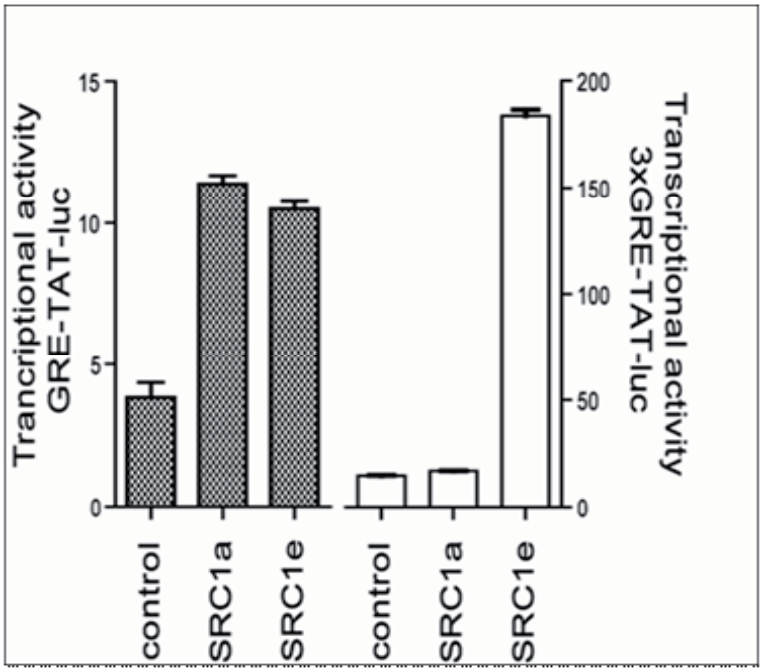


Fig. 2: Promoter-specific effects of steroid receptor coactivator splice variants 1a and 1e on the transcriptional activity of the glucocorticoid receptor. On a single GRE-containing promoter (filled bars) both overexpression of the SRC1 isoforms stimulate GR-driven transcription of the reporter gene.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

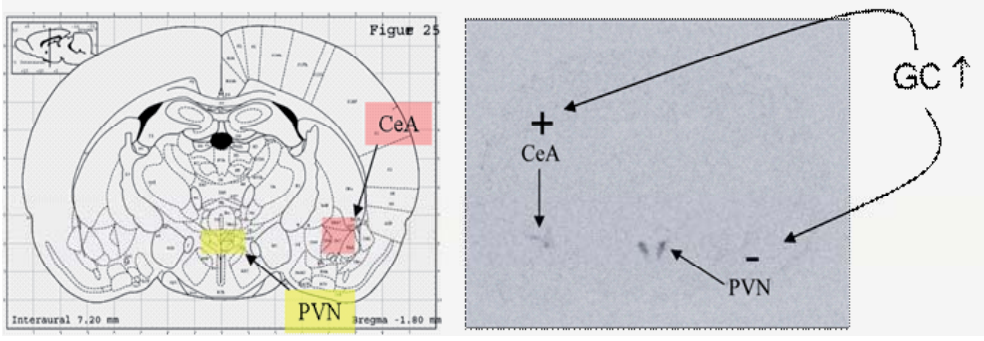


Fig. 3: Increase in glucocorticoid blood levels results in a decrease of CRH expression in the PVN, but concurrently stimulates CRH expression in the CeA in the rodent brain. The mechanism(s) by which glucocorticoids can exert cell-specific opposing effects on CRH gene expression in the rodent brain 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

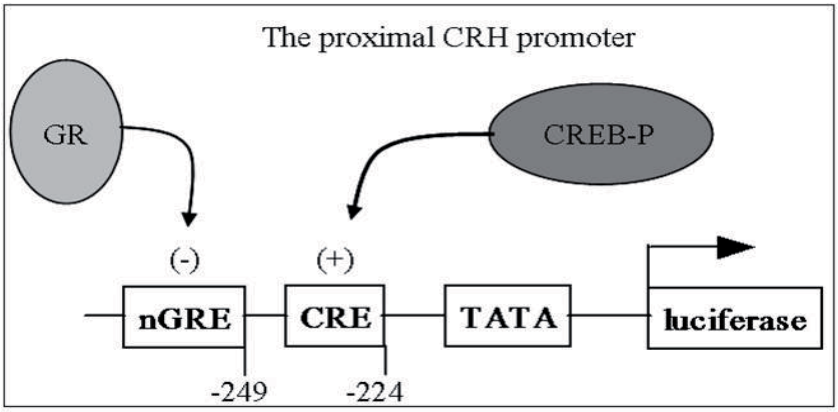


Fig. 4: Schematic overview of the two principal stimulatory or inhibitory inputs of the human proximal CRH promoter. The CRE and the nGRE located at respectively position -224 and -249 (relative to transcription start site = 0) are indispensable for the cAMP and glucocorticoids-induced effects on CRH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

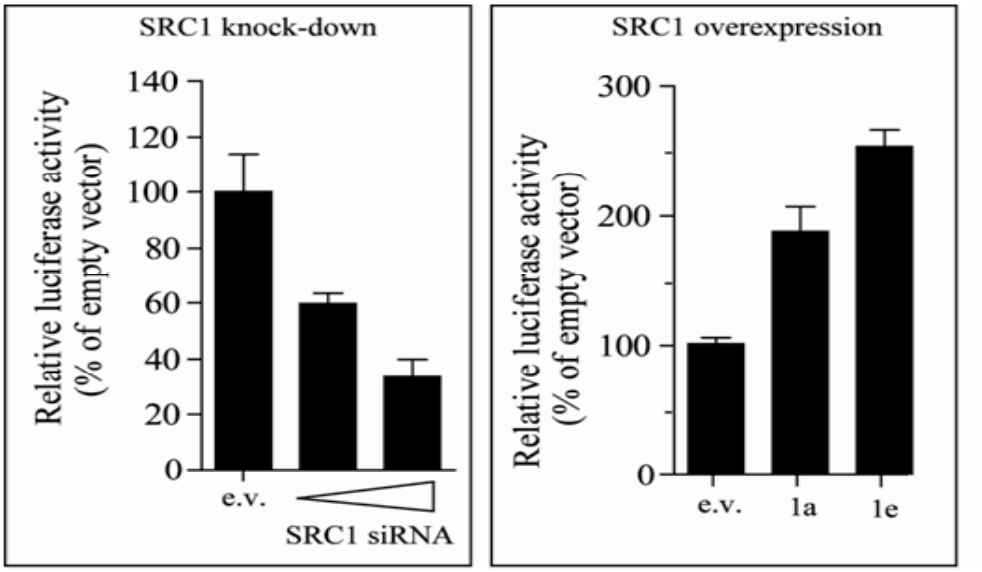


Fig. 5: FSK-induced CRH stimulation in transiently transfected AtT-20 cells. Specific SRC1 knock-down resulted in a loss of FSK-induced stimulation whereas SRC1a or SRC1e overexpression increased FSK-induced CRH gene expression.

(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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