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

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Chapter III

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

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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.

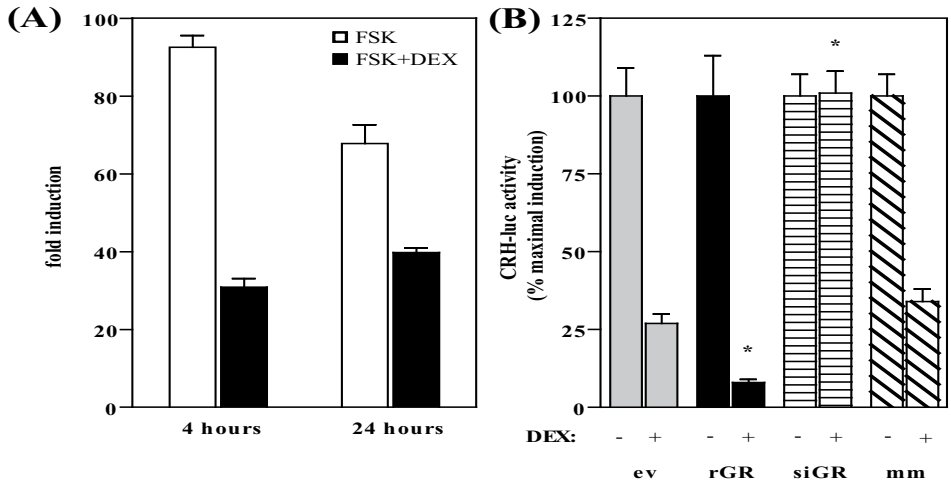

Fig. 1: GR-mediated repression of the forskolin-induced CRH-promoter activity.
(A) Dexamethasone-induced CRH repression after 4 and 24 hours co-treatment with forskolin. Data are expressed as the fold induction over basal activity of the promoter in untreated cells. The average values (n=4; ±SD) are 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

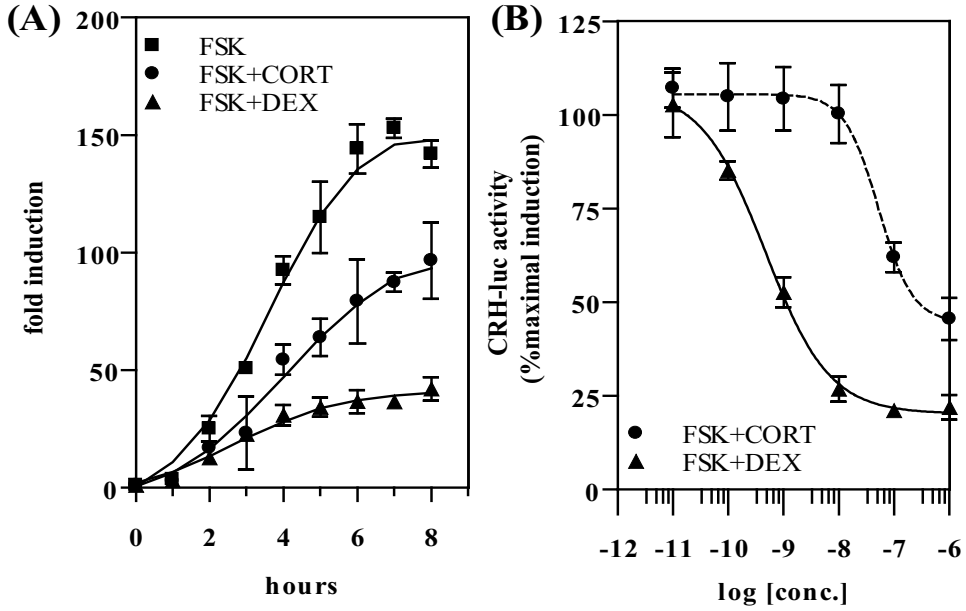

Fig. 2: Corticosterone-dependent repression of the CRH-induced gene.
(A) Time course of CRH-promoter activity after forskolin treatment w/wo dexamethasone or corticosterone co-treatment. The effect of forskolin (■), co-treatment with 10-7M DEX (▲) or 10-7M CORT (▼) on CRH-promoter activity are shown 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.

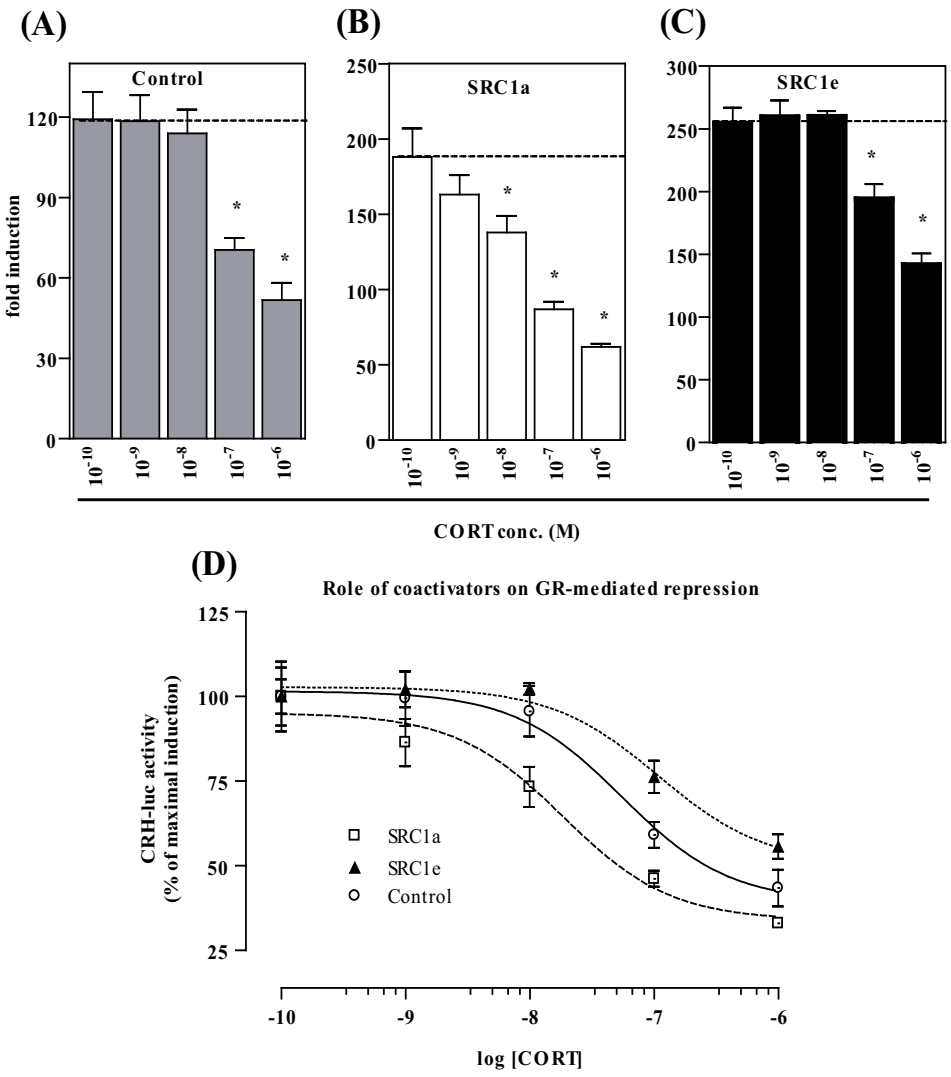

Fig. 3: Differential effect of SRC1 isoforms on the GR-mediated CRH repression.
CRH-promoter activity expressed as fold induction (normalised against basal activity of the promoter). The luciferase activity was measured after 4 hours incubation. The average values (n=4; ± SD) are shown. * indicates significantly 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.

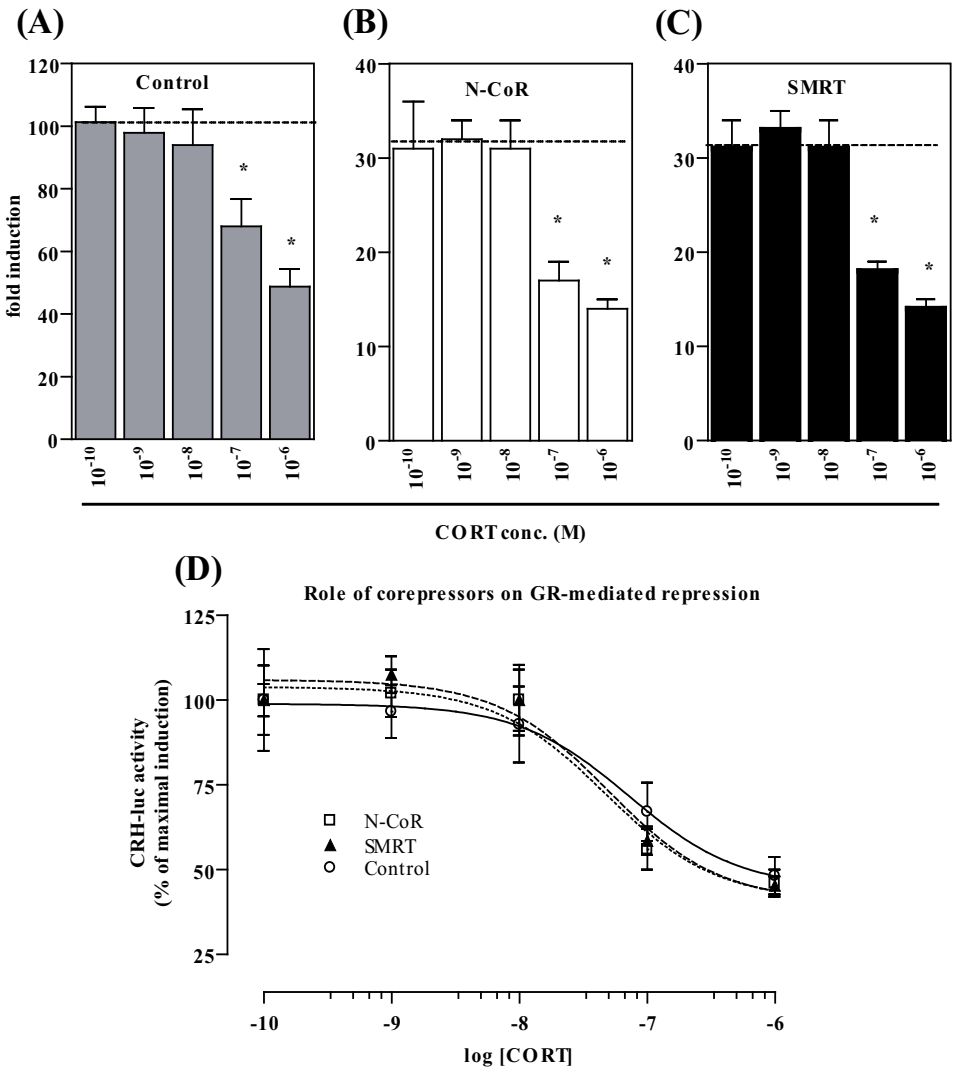

Fig. 4: Corepressors N-CoR and SMRT do not affect the GR-mediated CRH repression.
CRH-promoter activity expressed as fold induction (normalised against basal activity of the promoter). The luciferase activity was measured after 4 hours incubation. The average values (n=4; ± SD) are shown. * indicates 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).

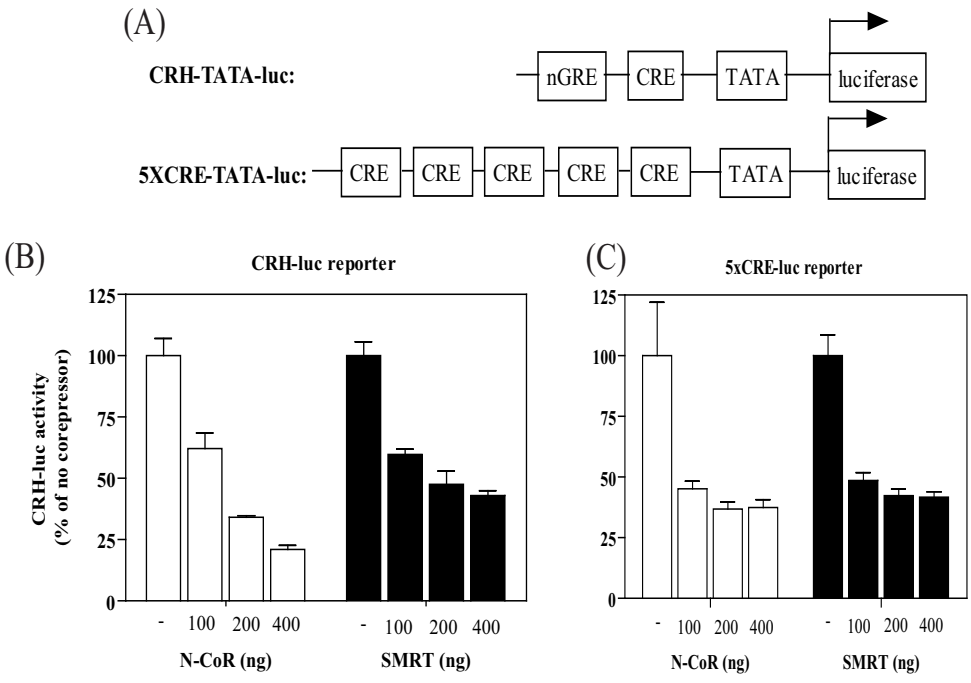

Fig. 5: CREB-mediated transcription is suppressed by N-CoR and SMRT.
(A) Schematic representation of the CRH-luciferase reporter, with both the negative GRE (nGRE) and the CRE, and the synthetic 5xCRE containing reporter. Dose-dependent effects of N-CoR and SMRT overexpression on the CRH-luc reporter (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.

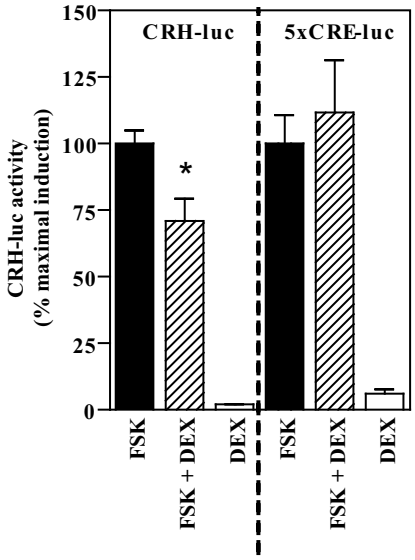

Fig. 6: Promoter specific effects of DEX co-treatment
Co-treatment with 10-7M DEX suppresses forskolin-induced CRH promoter activity whereas it does not affect the promoter activity of the forskolin-induced 5xCRE-containing promoter. The GR-mediated repression is promoter-dependent and is not likely 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.

5. Acknowledgments

This study was supported by NWO VIDI Grant 016.036.381 (O.C.M.) and the Royal Netherlands Academy for Arts and Sciences (E.R.d.K.). We thank Drs. Nicole Datson and Thomas Dijkmans for critical review of the manuscript.

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