Methylation at the CpG island shore region upregulates Nr3c1 promoter activity after early-life stress

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Abbreviations: Avp, arginine vasopressin; BPD, borderline personal disorder; CGI, CpG island; ChIP, chromatin immunoprecipitation; Crh, corticotropin releasing hormone; CUS, chronic unpredictable stress; Dusp1, dual specificity phosphatase 1; ELS, early-life stress; EMSA, electrophoretic mobility shift assay; Fkbp5, FK506 binding protein 51; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HPA, hypothalamic-pituitary-adrenal; MDD, major depressive disorder; Pomc, pro-opiomelanocortin; PTSD, posttraumatic stress disorder; PVN, paraventricular nucleus; Sgk1, serum glucocorticoid kinase 1; YY1, Yin Yang.

Early-life stress (ELS) induces long-lasting changes in gene expression conferring an increased risk for the development of stress-related mental disorders. Glucocorticoid receptors (GR) mediate the negative feedback actions of glucocorticoids (GC) in the paraventricular nucleus (PVN) of the hypothalamus and anterior pituitary and therefore play a key role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis and the endocrine response to stress. We here show that ELS programs the expression of the GR gene (Nr3c1) by site-specific hypermethylation at the CpG island (CGI) shore in hypothalamic neurons that produce corticotropin-releasing hormone (Crh), thus preventing Crh upregulation under conditions of chronic stress. CpGs mapping to the Nr3c1 CGI shore region are dynamically regulated by ELS and underpin methylation-sensitive control of this region’s insulation-like function via Ying Yang 1 (YY1) binding. Our results provide new insight into how a genomic element integrates experience-dependent epigenetic programming of the composite proximal Nr3c1 promoter, and assigns an insulating role to the CGI shore.

Introduction

Early-life adversity can elicit life-long increases in glucocorticoid (GC) secretion and disruption of the homeostatic mechanisms that regulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis.1 All of these events increase the risk for the development of stress-related diseases, including mood and affective disorders, anxiety disorders, borderline personal disorder (BPD), and posttraumatic stress disorder (PTSD).1,2

Epigenetic mechanisms are increasingly recognized for their role in the dynamic transduction of the effects of changing environments on the genetic blueprint.3 In this regard, DNA methylation has been recently shown to translate social experiences into long-lasting changes in gene expression and the manifestation of distinct phenotypes. This form of ‘molecular plasticity’ is thought to facilitate an organism’s capacity to mount an adaptive response through integration of multilayered gene-environment interactions.4

The glucocorticoid receptor gene (NR3C1) encodes a ligand-gated transcriptional regulator that controls endocrine responses to stress as well as metabolism, inflammation, and reproduction.5 The structure of NR3C1 is highly conserved between human, rat,6 and mouse;7 strong homologies are also found in multiple 5’ untranslated exons in the proximal promoter regions, which produce various mRNA isoforms encoding the same protein.8 Pioneer studies in the rat showed that persistent changes in exon 17 DNA methylation occur as a function of quality of maternal care9; subsequently, childhood trauma,10-14

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HPA-axis activity and hypometabolism remain unclear. Although the cause-effect relationships of these observations remain unclear.

We previously showed that ELS in mice causes sustained HPA-axis activity and hypometabolism of the hypothalamic arginine vasopressin (Avp) gene and pituitary pro-opiomelanocortin (Pomc) gene. Here, we report that ELS induces site-specific hypermethylation of an Nr3c1 control element, which coordinates expression of multiple GR transcripts and overall GR protein in Crh-expressing neurons and thus prevents upregulation of Crh under conditions of chronic stress in adulthood.

**Results**

**ELS upregulates hypothalamic GR expression**

In contrast to changes in Avp expression, which appear within days of exposure to ELS, increases in hypothalamic GR levels are delayed, becoming first detectable after termination of the stressor. However, like those of Avp, the changes in GR expression persisted for at least 6 months (Fig. 1A). Notably, GR expression was not altered in the hippocampus and pituitary, both GR-responsive tissues that are prominent GC negative feedback sites (Fig. 5A).

**Nr3c1** contains multiple 5′untranslated exons in its promoter region that can give rise to various mRNA isoforms. Although differentially regulated and expressed, these isoforms can be translated into the same protein. Here we show that multiple GR transcripts derived from the proximal Nr3c1 promoter region are upregulated in the PVN of 3-month-old ELS-treated mice (Fig. 1B), consistent with the observed net increase in total GR transcript levels (Fig. 1A).

**Increased GR expression translates into higher GR transactivation**

Corticosterone injections in ELS mice resulted in higher induction of several GC-responsive genes—Fkbp5 (FK506 binding protein 51), Dusp1 (dual specificity phosphatase 1), and Sgk1 (serum glucocorticoid kinase 1)—indicating enhanced transcrip-
tional activity of ELS-upregulated GR (Fig. 1C). These target genes play a crucial role in GC signaling, but also in neuronal plasticity, and encode a maximum of 2 glucocorticoid response elements (GREs). Results from in vitro ChIP experiments on chromatin derived from microdissected PVN of corticosterone-treated ELS mice revealed higher GR occupancy at the intronic glucocorticoid response element (GRE) of Fkbp5 and at one of the 2 GREs of Dusp1 (GRR-29) (Fig. 1D).

The above analysis shows that ELS results in a site-specific upregulation of multiple GR transcripts, a net increase in receptor mRNA, and enhanced transcriptional regulation of target genes.

**ELS induces persistent hypermethylation at the CGI shore in Nr3c1**

Computational analysis predicted one major and 2 minor CpG-dense islands (CGIs) within the proximal promoter of the mouse Nr3c1 (Fig. 2A). Since ELS upregulates the expression of multiple GR transcripts, the entire proximal promoter was investigated for experience-induced changes in DNA methyla-
tion. This analysis revealed only sparse methylation in ELS-naive mice (Fig. 2A), consistent with the view that promoter CGIs, especially those with high CpG content, generally escape DNA methylation. Also, no significant differences in DNA methylation of the mouse Nr3c1 proximal promoter (including the rat homolog of exon 1,7 previously shown to respond to differences in the quality of maternal care), were found in PVN tissues from control and ELS mice (Fig. S2).

On the other hand, a cluster of moderately methylated CpGs was found adjacent to the most distal CGI (Fig. 2A), a topographic region called “CGI shore.”

**Methylation-sensitive YY1 binding to the Nr3c1 CGI shore region**

DNA methylation is thought to hinder binding of transcription factors to DNA and, at the same time, to favor the recruitment of protein complexes that promote an inactive chromatin structure. Computational analysis of the CGI shore region predicted a canonical binding site for the multifunctional and ubiquitously-expressed zinc finger transcription factor YY1 which straddles the ELS-responsive residue CpG3. As in the human Nr3c1 promoter, 2 additional YY1 binding sites are present in the CGI, but these either do not contain a CpG dinucleotide or are poorly methylated and unresponsive to ELS (Fig. S2).

**Binding of YY1 at the shore region was assessed by electrophoretic mobility shift assays (EMSA) on nuclear extracts from YY1-transfected cells and using oligonucleotides spanning either the**
YY1 occupancy at the CGI shore represses *Nr3c1* transcription in an ELS-responsive mode

Our finding that GR expression simultaneously increases with CpG3 hypermethylation (Figs. 1A; 2B) and methylation-sensitive YY1 binding to CpG3 (Fig. 3) suggests that YY1 occupancy of this region confers transcriptional repression. Previous work has shown that, depending on the cellular and promoter context and available binding partners, YY1 can act as either an activator or repressor of transcription. Here, transfection assays in hypothalamic N6 cells revealed that insertion of the CGI shore region reduces promoter activity by 40% compared to the parent vector and that this effect can be partly reversed by a point mutation of CpG3 at the YY1 binding site (Fig. 4A). Similarly, knockdown of YY1 in N6 cells enhanced reporter activity, strengthening the evidence that YY1 has a repressor function at the CGI shore region (Fig. 4B; Fig. S6).

To directly assess YY1's transcriptional function at the CGI shore, we conducted sequential in vivo ChIP experiments, using antisera directed against histone modifications and proteins (namely, H3ac, H3K27me3, H3K9me2, Hdac1, Hdac2, Suz12, Ezh2, and Ehmt2/Ehmt1 (alias G9a/GLP), which interacts with Kdm5a (alias Jarid1a) that associate and interact with YY1 to maintain transcriptional repression. Besides observing co-precipitation of YY1 with the repressive histone marks H3K9me2 and H3K27me3 at the *Nr3c1* CGI shore, we also found that Ehmt2, Kdm5a, and Hdac2 co-precipitate with YY1. These findings suggest that these
proteins may contribute to the repression of Nr3c1 through post-translational modifications of histones (Figs. 4C-D).

The fact that ELS-induced upregulation of multiple GR transcripts in the PVN of ELS mice (Fig. 1B) raised the possibility that the CGI shore region regulates transcription across the entire proximal Nr3c1 promoter and, furthermore, that YY1 binding might affect this function. We therefore next investigated whether the CGI shore region shields Nr3c1 promoter activity from upstream regulatory influences. Using a well-established assay system, we detected an insulation-like activity of the CGI shore region (Fig. 4E). Notably, this control function was largely abolished after mutation of the YY1 DNA-binding site, consistent with the results of other canonical Nr3c1 target genes (Fig. 1C). The lack of effects on Crh are consistent with the results of other studies showing that basal or stimulated Crh transcription in vivo are barely affected by alterations of the corticosterone milieu over a broad range of concentrations.

The parvocellular division of the PVN harbors distinct sub-populations of peptidergic neurons, among them neurons that express only Crh or Avp, or both.66-68 Here we used triple fluorescence immunohistochemistry to identify which subsets of PVN neurons express GR. GR expression was confined to Crh neurons in control mice and ELS mice, although expression levels were significantly higher in the latter group (Fig. 5B; Fig. S7).

Corticosterone application failed to repress Crh in ELS and control mice (Fig. 5A), whereas acute stress triggered an enhanced glucocorticoid response in ELS mice (Fig. S8). Together, these findings suggest that higher GR expression in the PVN of ELS mice does not inhibit HPA-axis activity following acute surges in glucocorticoids. This prompted us to investigate the consequences of ELS-induced GR upregulation on Crh expression after exposing animals with a history of ELS to chronic stress in adulthood. Since chronic stress has been consistently reported to upregulate hypothalamic Crh mRNA expression in adult rodents, we predicted results that would reflect enhanced inhibitory control of Crh in ELS mice because of their higher levels of GR expression (cf. Fig. 1A), i.e., Crh mRNA levels would be higher in non-ELS animals than in ELS-treated animals when exposed to a chronic unpredictable stress (CUS).
paradigm. As shown, however, Crh expression was attenuated in ELSxCUS vs. ELS mice (Fig. 5C); this is remarkable because mice with a history of either ELS alone or ELSxCUS expressed significantly higher levels of GR mRNA as compared to control and CUS animals (Fig. 5C); again, GR expression was restricted to Crh neurons in CUS and ELSxCUS mice (Fig. 5B; Fig. S7). Moreover, consistent with these Crh expression profiles, ELSxCUS and control mice showed similar levels of blood corticosterone at 30 min after an acute stressor, responses that were significantly lower than those observed in animals exposed to either ELS or CUS alone. In addition, baseline corticosterone levels were fastest restored to baseline after the acute stressor in ELSxCUS mice, indicative of their more efficient GC negative feedback mechanisms (Fig. 5B).

Taken together, these data show that ELS occludes the normal upregulation of Crh expression when mice are exposed to chronic stress during adulthood.

**Discussion**

The present work shows that ELS programs Nr3c1 expression by site-specific hypermethylation at the CGI shore in parvocellular Crh-positive neurons of the hypothalamus and prevents Crh upregulation by subsequent exposure to chronic stress.

Most studies on epigenetic programming of GR expression have focused on the promoter region upstream of exon 1 in (rat), 1F (human) or the mouse orthologous region; in addition, some authors have reported changes in the expression of multiple GR transcript variants. Although a large proportion of the Nr3c1 locus appears subject to epigenetic regulation, genomic regions that regulate multiple Nr3c1 promoters within the CGI have not been described hitherto.

Recent in-depth methylation analysis of the whole genome in diverse human tissues showed that only approximately 20% of autosomal CpGs are subject to dynamic epigenetic modifications. These residues localize more distal to the regions that are usually studied and presumably harbor genomic elements involved in tissue-type differentiation. This study also revealed that CGI shores, which are often differentially methylated in tissues derived from different lineages, were among those genomic regions that showed the greatest variation in epigenetic marking during normal development.

Two key findings of the present study are that CpGs mapping to the Nr3c1 CGI shore region i) underpin methylation-sensitive control of this region’s insulin-like function via YY1 binding and ii) are dynamically regulated by ELS. Collectively, our results provide new insight into a genomic element integrating experience-dependent epigenetic programming of the composite proximal Nr3c1 promoter and assign to the CGI shore a new role in insulation.

Administration of corticosterone led to transactivation of a set of canonical GR target genes in the hypothalamus of ELS mice, indicating the transcriptional potency of the GR upregulated by ELS. The genes activated in this paradigm included Fkbp (role in an intracellular feedback loop terminating GR function), Sgk1 (promotes nuclear localization of GR and prolongs its activation in the absence of GC, upregulates ion channels, enzymes, and transcription factors that modulate hormone release, neuroexcitability, and cell proliferation) and the serine-threonine phosphatase Dusp1, alias MKP-1 (triggers a decrease in phosphorylation-activated ERK1/2 MAPKs and CREB-dependent transcription of brain-derived neurotrophic factor). The
products of all of these genes are implicated in stress-related affective disorders,\textsuperscript{28,29,54} however, it is unclear as to whether they play a role in the hypothalamic control of ELS-induced stress responses.

Given that CRH is the major neuropeptidergic driver of the endocrine response to stress and that chronic stress (CUS) during adulthood upregulates hypothalamic Crh expression (Fig. 5C), the finding that Crh expression was not elevated in adult mice with a history of ELS was unexpected (Fig. 5A; C). These divergent responses most likely reflect the recruitment of specific physiological mechanisms by the different types of stressor, context and age at which they were imposed.\textsuperscript{2,55,56} This interesting observation suggests that ELS buffers against subsequent lifetime stressors, a phenomenon also recently reported in rodents and humans.\textsuperscript{57-59}

Glucocorticoids inhibit hypothalamic synthesis and secretion of hypothalamic CRH, their actions being mediated by GR.\textsuperscript{5} Intriguingly, ELS and ELSxCUS mice showed control-like levels
of Crh expression despite their contemporaneously upregulated levels of GR (Fig. 5C). Nevertheless, ELS mice were still able to secrete increased amounts of corticosterone (ELS > control and ELSxCUS) when exposed to an acute stressor (Fig. S8). This result indicates that the integrity of the neural mechanisms governing pituitary-adrenal function are maintained in ELS mice and hints at compensatory mechanisms governing HPA-axis function in these animals. In contrast, the finding of control-like corticosterone secretion in ELSxCUS animals suggests that the initial stressor (ELS) attenuates the impact of subsequent CUS through mechanisms that still await elucidation.

Since stressors and the response they elicit are context-dependent and differ in quality as alluded to above, we examined whether Crh expression in ELS mice is subject to inhibition by exogenous corticosterone; the latter paradigm bypasses the complex pathways activated by stress. Contrary to expectation, especially in light of the above-mentioned corticosterone-driven transactivation of GR in ELS mice (Fig. 1), injection of corticosterone failed to significantly suppress Crh expression (Fig. 5A). This unanticipated finding concurs with another recent report. Together with the present observation that GR-mediated negative feedback is less efficient (delayed) in ELS mice (Fig. S8), these findings suggest that GR regulation of the Crh gene (specifically, in contrast to Fkbp, Sgk1, and Dusp1) is persistently altered by ELS, the molecular underpinnings of which warrant future analysis. One possibility is that enhanced GR expression in ELSxCUS mice facilitates formation of repressive chromatin complexes at the Crh promoter through direct or long-range interactions with enhancer elements.

The long-term effects of early-life experiences appear to be stimulus-specific and dependent on the brain areas that perceive and process the respective stimuli by coordinating downstream cellular and molecular responses, including activation of the epigenetic machinery. In this respect, pioneering studies on differences in the quality of maternal care evidenced hypomethylation of Nr3c1 at exon 1 in the hippocampus and enhanced binding of the transcriptional activator NGFI-A. In contrast, we show here that early-life adversity resulted in hypermethylation of Nr3c1 at
the shore region in Crh-positive neurons and impaired binding of the transcriptional repressor YY1. Taken together, these findings suggest that epigenetic programming in response to early-life experiences leads to tissue- and cell-type specific effects on Nr3c1 methylation and subsequent alterations in the binding of regulatory proteins conferring transcriptional regulation of Nr3c1.

**Materials and Methods**

**Animal treatments**

Maternal separation was used to induce ELS in male C57Bl/6N mice, as described elsewhere. Two slightly modified version of a uCDS protocol was used. Two-month-old mice received individual stressors that alternated in order and which were applied at unpredictable times of the day over a period of 4 weeks [during the inactive phase: 1 h shaking of 3–4 mice in a 10 × 5 cm box at low frequency (~60 rpm), 30 min restraint stress, 1 h white noise exposure (80 dB) of 3–4 mice placed in a 10 × 5 cm box; during the active phase for 12 h: tilted cage in a 45° angle, once per week illumination, damped bedding].

Mice were acutely stressed by placing them cage-wise in a plastic box (10 × 10 × 5 cm) that was fixed on a vibrating platform (25 Hz) for 2 minutes. Ten-week old mice received intraperitoneal injections of either saline or corticosterone (0.1, 1.0 or 10.0 mg/kg in 25% cyclodextrin [Sigma-Aldrich, Deisenhofen, Germany]) at the trough of endogenous corticosterone secretion. All procedures on animals were approved by the Regierung von Oberbayern and were in conformity with European Union Directive 2010/63/EU.

**In situ hybridization (ISH), quantitative PCR, and bisulfite sequencing**

GR transcripts were detected as described. Reverse transcribed RNA was quantified using a LightCycler 2.0 and LightCycler FastStart DNA Master plus SYBR Green I Kit (Roche, Mannheim, Germany). Relative expression was calculated using the ΔΔCt method with efficiency correction as described. Methylation analysis of genomic DNA (100–200 ng) was performed as described. Primers used for qPCR and for bisulfite sequencing are listed in Tables S1 and S2.

**Plasmids, cell culture, and transfections**

Complementary pairs of single stranded DNA oligomers (Table S3) containing the wild type (wt) or mutated (mut) distal YY1 binding element of the Nr3c1 promoter were cloned into the BamHI and Neol site of pBluescript II SK vector. Transcriptional activity of the CGI boundary sequence (−4,770 to −4,554 bp relative to start codon ATG) was tested in the Gaus- sia-luciferase (GLuc) expression vector pG3GLuc. Further details on the generation of constructs described in this manuscript are available on request.

Mouse hypothalamic N6 and N44 cells (Cedarlane CELLu-
tions, Burlington, Canada), culture conditions and 5-azacytidine treatment were described elsewhere. N6 cells (2 × 10⁵) were transfected with 4 µg plasmid DNA and 6 µl Turbofect (Thermo Scientific, Schwerte, Germany) per well of a 6 well plate. Relative light units were normalized against β-galactosidase activity from a cotransfected β-galactosidase expression vector. Pilot experiments evidenced an efficient knockdown of YY1 mRNA in N6 cells by 50 pmol of YY1 siRNA without obvious effects on viability. Accordingly, 50 pmol YY1 or scramble siRNA (Eurofins MWG Operon, Ebersberg, Germany) were cotransfected with 1 µg GLuc expression vector and 5 µl Lipofectamine 2000 (Life Technologies, Darmstadt, Germany). Sequences were: YY1 sense (5′-ucaggugagagauuuac-3′) and antisense (5′-agagacagucuuuccgu-3′) and scramble siRNA sense (5′-aggagugauugcgcguu-3′) and antisense (5′-caggag-
gauacacacuu-3′). Cells were harvested for analysis 24–48 h post transfection.

**In vitro methylation**

Wild type and mutated YY1 DNA-binding sites were cloned into pBSK vectors and sequence verified constructs were in vitro methylated using CpG methyltransferase SsI (New England Biolabs, Frankfurt am Main, Germany) according to man-
ufacturer’s instructions. Unmethylated DNA was treated the same, but without SsI. Following phenol/chloroform extraction, completeness of methylation was controlled by digesting both methylated and unmethylated plasmids using methylation-sensitive restriction enzyme Taul.

**Electrophoretic mobility shift assays (EMSA)**

Double stranded oligonucleotides were obtained by HindIII and Neol digestion of the corresponding pBSK vector constructs. Fragments were labeled with [α-32P]-dCTP and DNA Polymerase I (Klenow) (New England Biolabs, Frankfurt am Main, Germany). Electrophoresis of LLC-PK1 cells [ATCC CL-101] was used to efficiently express an YY1 expression vector (pCMV-
YY1). About 4 µg of nuclear extract was incubated alone or with an access of competitor consensus oligonucleotide (yy1), its mutated form (yy2) and with YY1- or pCAF-specific antibody under binding conditions described elsewhere. Thereafter, 1 µl of 32P-labeled duplex probes (20,000 cpm) was added to each reaction mix and incubated for 20–25 min at RT. The reaction mixtures were electrophoresed for 1–2 h at 100 V at 12°C in 0.5 x TBE and dried gels were exposed to an MS autoradiography film (Sigma-Aldrich) for 7–10 h at ~80°C.

**Chromatin immunoprecipitation (ChIP)**

ChIP and sequential ChIP experiments on 2–3 × 10⁶ N6 cells and PVN punches from 2- to 3-month old mice were performed as described elsewhere. ChIP-qPCR values were normalized relative to the IgG-antibody control (fold enrichment). Relative enrichment was calculated by normalization of fold-enrichment data against the average fold-enrichment of salinetreated control mice. Primers used for ChIP-qPCR analysis are listed in Table S4.

**Immunohistochemistry, image acquisition**

Brains of 3-month-old mice were PFA-fixed and sucrose cryo-
preserved. Cryostat sections (30 µm) were thawed, re-fixed in
4% PFA and blocked (5% donkey normal serum, 5% BSA and 0.2% Triton X-100). Primary antibodies were applied for 16 h at 12°C and secondary antibodies for 2 h at room temperature. DAPI staining (10 μg ml⁻¹, Sigma-Aldrich) was performed for 2 min. Primary and secondary antibodies used for triple-staining of GR, Avp and Chr are listed in Table S5. Images were obtained with an Olympus IX81-FV1000 laser-scanning confocal microscope; images (1,024 × 1,024 pixels) were adjusted uniformly for brightness and contrast using FV10-ASW 2.0 software (Olympus).

Antibodies, YY1-specific antisera, immunoblotting
Antibodies are listed in Table S5. YY1 antisera (acc: NP_033563) were raised by Pineda Antibody-Service by injecting New Zealand White rabbits with a GST-conjugated peptide (NP_033563) were raised by Pineda Antibody-Service by inject-

4% PFA and blocked (5% donkey normal serum, 5% BSA and 0.2% Triton X-100). Primary antibodies were applied for 16 h at 12°C and secondary antibodies for 2 h at room temperature. DAPI staining (10 μg ml⁻¹, Sigma-Aldrich) was performed for 2 min. Primary and secondary antibodies used for triple-staining of GR, Avp and Chr are listed in Table S5. Images were obtained with an Olympus IX81-FV1000 laser-scanning confocal microscope; images (1,024 × 1,024 pixels) were adjusted uniformly for brightness and contrast using FV10-ASW 2.0 software (Olympus).

Antibodies, YY1-specific antisera, immunoblotting
Antibodies are listed in Table S5. YY1 antisera (acc: NP_033563) were raised by Pineda Antibody-Service by injecting New Zealand White rabbits with a GST-conjugated peptide corresponding to the amino acids 1–54. Characterization of YY1-specific antisera was performed by transfection of pRK7-FLAG-Y1 or an equal amount of filling plasmid into LLC-PK1 cells. Whole cell protein extracts (20 μg) were tested either with monoclonal FLAG-specific M2 antibody (Sigma-Aldrich) or with YY1-specific antibodies (custom-made, CM1, CM2; H-414 X Santa Cruz Biotechnology, Heidelberg, Germany). Performance of YY1-specific antisera (CM1) in ChIP experiments was characterized in N44 cells by using the commercial YY1-specific antibody (H-414 X), a negative IgG control, positive and negative control primer sets for verification of enrichment.

Whole cell extracts (50 μg) from PVN punches (2 mice per condition) were immunoblotted and tested with the commercial YY1-specific antibody (H-414 X) or an antibody against β-actin (D6A8 Cell Signaling, Frankfurt/Main, Germany), which served as a loading control.

Insulation assay
Constructs linearized by Sall digest (0.5 μg) were electroporated into 5 × 10⁶ K562 cells [ATCC CCL-243], pNI and pNI-CD plasmids were served as controls. After 24 h recovery in Improved MEM zinc option (Life Technologies) cells were plated in 0.3% soft agar with 0.9 mg ml⁻¹ G418 sulfate (Merck Millipore, Schwalbach, Germany). Viable colonies were stained with methylthiazolyldiphenyl-tetrazolium (MTT) after 2 weeks of GR, Avp and Crh are listed in Table S5.

In silico analysis, statistics
Embass CpGplot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) was used to identify CGIs. Transcription Element Search System (TESS) was used to predict the YY1 binding site covering CpG3 (http://www.ncbi.nlm.nih.gov/gi-bin/seqg ).

Quantitative data were analyzed using SPSS software (IBM, Munich, Germany). All numerical data were expressed as mean ± SEM (standard error of the mean). Statistical evaluation was basically performed with analyses of variance (ANOVA s or MANOVA s) followed by univariate F-tests for testing simple effects or contrasts. By multivariate variance analyses main or interaction effects were tested about significance with Wilks multivariate F-tests or averaged F-tests, the last one especially in cases of small samples. GR expression data at different ages and fold-induction data on GR target genes were transformed by using artnan transformation to keep variance homogeneity. Two-tailed paired or unpaired student’s t test was used to compare mean differences. One sample t-test was used to compare means to normalized controls of in vitro experiments. An α = 0.05 was accepted as nominal level of significance. All post hoc tests were performed at an adjusted (according to Bonferroni) level of significance, in order to keep the type I error lower or equal to α.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this paper can be accessed on the publisher’s website.

References


