

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

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Keywords: CpG island shore, DNA methylation, early-life stress, glucocorticoid receptor, insulator, Yin Yang

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 Yin 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 mouse⁷; 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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Submitted: 11/04/2014; Revised: 01/27/2015; Accepted: 01/30/2015

<http://dx.doi.org/10.1080/15592294.2015.1017199>

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suicide,^{15,16} BPD,¹⁷⁻¹⁹ and PTSD²⁰⁻²² were shown to produce similar epigenetic modifications. While most of the latter studies focused on exon 1F, the human homolog of 1₇, some authors reported upregulation of multiple transcripts after early-life stress (ELS).^{15,16,22} Comprehensive analysis of the *NR3C1* gene in rat²³ and human²⁴ demonstrated broad changes in DNA methylation, histone modifications, and upregulation of multiple exon 1 transcripts in individuals that had experienced adverse events during early life, although the cause-effect relationships of these observations remain unclear.

We previously showed that ELS in mice causes sustained HPA-axis activity and hypomethylation of the hypothalamic arginine vasopressin (*Avp*)²⁵ and pituitary pro-opiomelanocortin (*Pomc*) gene.²⁶ 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,²⁵ 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. S1).

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.^{7,8} 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 transcriptional activity of ELS-upregulated GR (Fig. 1C). These target genes play a crucial role in GC signaling, but also in neuronal plasticity,²⁷⁻³⁰ and encode a maximum of 2 glucocorticoid response elements (GREs).³¹⁻³³ Results from *in vivo* 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). In summary,

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)²⁵ 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 methylation. 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.²⁵ Also, no significant differences in DNA methylation of the mouse *Nr3c1* proximal promoter (including the rat homolog of exon 1₇, previously shown to respond to differences in the quality of maternal care),⁹ 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.”³⁴ Shore regions show a lower CpG content compared to canonical CGIs and frequently undergo DNA methylation.³⁴ Tissue-specific differential methylation was found in CpG1-3 at the *Nr3c1* shore region in samples originating from different embryonic lineages (Fig. S3), a finding consistent with the view that CGI shore regions contribute to tissue-specific differences in gene expression.^{34,35} Accordingly, we were prompted to investigate whether ELS programs *Nr3c1* by targeting its shore region.

Methylation of CpGs increased with age (specifically, at CpGs 2 and 3 in 6-week old ELS mice, and CpGs 3, 7 and 9 in 3-month old ELS mice). Importantly, hypermethylation of CpG3 was robustly maintained (Fig. 2B) and resulted in gradual overall hypermethylation and age-related increases in GR mRNA expression in ELS mice (Figs. 1A; 2C).

The above results demonstrate that mouse *Nr3c1* contains a CGI shore region that can be differentially methylated in a tissue-specific fashion; this CGI shore region serves as a template for ELS-induced hypermethylation.

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.⁴ 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,³⁶ 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

wild type sequence or a mutation of the ELS-responsive CpG3 residue. Specific and strong binding of YY1 to the wild type sequence was revealed by competition and supershift experiments (Fig. S4A). In addition, mutational analysis demonstrated that YY1 binding critically depends on the central CpG3 dinucleotide (Fig. S4B). Confirming the importance of CpG3 in methylation-sensitive YY1 binding, *in vitro* methylation of wild type and CpG2 mutated oligonucleotides impaired YY1 binding to similar extents (Fig. 3A).

The GR-expressing N6 mouse hypothalamic cell line displays a methylation profile at the shore region that, with the exception of CpG1, resembles the one observed in the mouse PVN (Fig. 3B). Treatment of these cells with 5-azacytidine, a potent inhibitor of DNA methylation, reduced the level of methylation at the CGI shore (Fig. 3B) and concomitantly increased YY1 occupancy, as evidenced by ChIP experiments (Fig. 3C) in which an antiserum specifically directed against YY1 was used (Fig. S5). Bisulfite sequencing of YY1-immunoprecipitated DNA recovered from the PVNs of ELS-naïve mice corroborated methylation-sensitive DNA binding of YY1 *in vivo* (Fig. 3D). These observations indicate that low CpG methylation levels favor YY1 binding to the CGI shore region.

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.³⁷ 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).

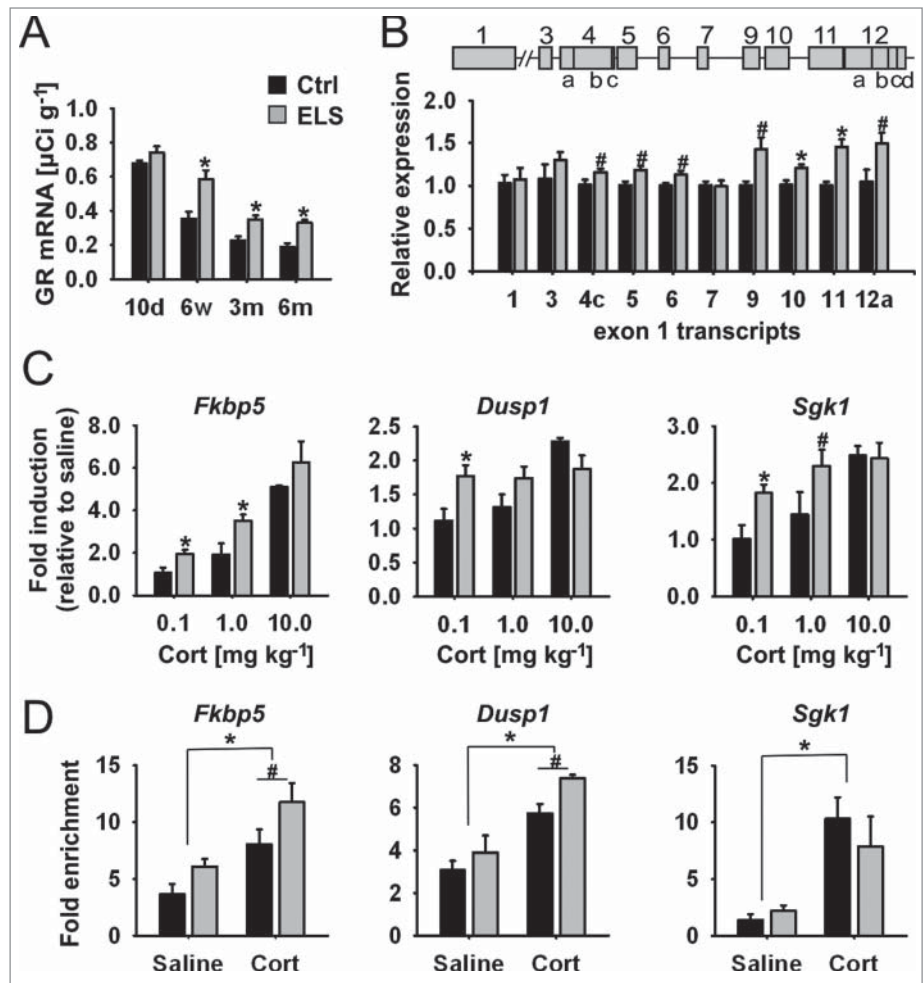


Figure 1. Increased GR expression translates to higher GR transactivation in ELS mice. (A) GR mRNA expression detected by ISH in PVN in mice aged 10 days, 6 weeks, 3 and 6 months (interaction of ELS and age; $P = 0.043$ by 2-way ANOVA; $*P < 0.0001$ by univariate F-tests; $n = 4-7$). (B) Map of the distal (1) and proximal (3 to 12) untranslated exon 1 of the mouse *GR* promoter region (upper panel). Regulation of GR exon 1 transcripts detected by qPCR in PVN of 3-month old control (Ctrl) and ELS mice. Expression data are normalized to *Atpj5* and relative to the expression of the Ctrl group (ELS effect; $P < 0.0001$ by one-way MANOVA and aver. F-test; $*P < 0.005$, $^{#}P < 0.05$ by univariate F-tests; $n = 7-9$). (C) Intraperitoneally injected corticosterone (Cort; 0.1, 1.0, 10.0 mg/kg) induced GR-responsive genes in PVN of Ctrl and ELS mice as measured by qPCR. Expression data are normalized to *Atpj5*; fold-induction is shown (interaction of ELS and dose; $P = 0.006$ by 2-way MANOVA; $*P < 0.017$, $^{#}P < 0.05$ by analysis of simple effects; $n = 4-8$). (D) GR occupancy of regulatory regions of GR target genes analyzed by *in vivo* ChIP (effect of corticosterone treatment, $P < 0.0001$; effect of ELS, $P = 0.058$ by 2-way MANOVA and average F-test; $*P \leq 0.001$, $^{#}P < 0.05$ by univariate F-tests; $n = 4-5$ ChIPs per group, PVNs of 3 mice were pooled per ChIP analysis). Data are means \pm SEM.

To directly assess YY1s transcriptional function at the CGI shore, we conducted sequential *in vivo* ChIP experiments, using antisera directed against histone modifications and proteins [namely, H3ac,³⁸ H3K27me3, H3K9me2,^{39,40} Hdac1, Hdac2,⁴¹ Suz12,⁴² Ezh2,³⁹ and Ehmt2/Ehmt1 (alias G9a/GLP),⁴⁰ which interacts with Kdm5a (alias Jarid1a)]⁴³ 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

Adult chronic stress differentially regulates hypothalamic Crh expression

The PVN is a major site of GC-mediated negative feedback regulation through Avp and Crh, the primary neuropeptidergic drivers of the HPA axis.⁵ Since GR expression was upregulated by ELS, we were prompted to compare the effects of an acute injection of corticosterone on Crh expression in ELS vs. control mice. While corticosterone did not significantly inhibit Crh mRNA expression in either treatment group (Fig. 5A), it should be recalled that corticosterone nevertheless induces 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.⁴⁵

The parvocellular division of the PVN harbors distinct sub-populations of peptidergic neurons, among them neurons that express only Crh or Avp, or both.⁴⁶⁻⁴⁸ 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 mice²⁵ (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,⁵ 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)

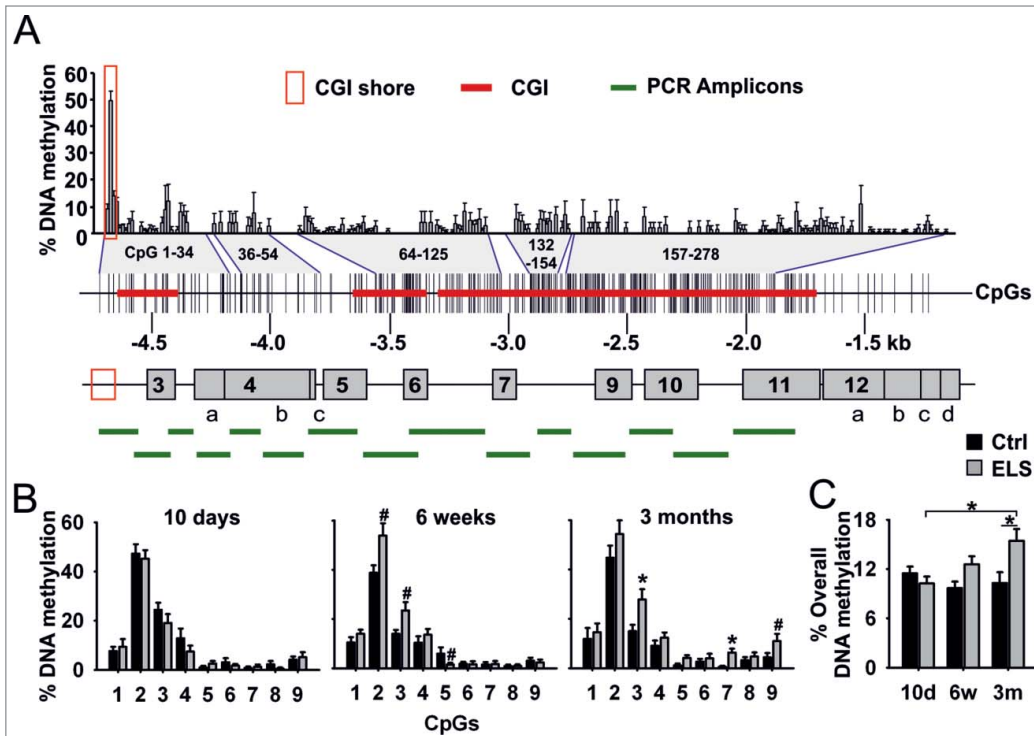


Figure 2. Methylation of proximal *GR* promoter and ELS-induced hypermethylation at CGI shore. (A) Map of the proximal untranslated first exons (gray boxes) of the mouse *Nr3c1* gene. CpG density, CGIs (red line) and experimentally determined CpG methylation in PVN are shown. Positions within the CpG panel are bordered by blue lines. Methylation of CpGs of the *GR* promoter region of 3-month old mice was analyzed by sequencing of 15 Alicons (green lines). Moderate CpG methylation was detected at CGI shore region (red box). CpGs are numbered, starting at CpG 1 at -4,732 base pairs (bp) relative to the ATG start codon. (B) Individual methylation of CpG1-9 in PVN of Ctrl and ELS mice aged 10 days, 6 weeks and 3 months (interaction of ELS and age; $P = 0.001$ by 2-way MANOVA and average F-tests with significance for CpG3, CpG5 and CpG7; ELS effects; $*P < 0.006$, $\#P < 0.05$ by univariate F-tests; $n = 9-10$ mice per age). (C) Overall methylation across 9 CpGs at the CGI shore in PVN (interaction of ELS and age; $P = 0.013$ by 2-way ANOVA; $*P < 0.05$ by univariate F-tests). Data are means \pm SEM.

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,⁴⁴ 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, supporting the view that YY1 plays a critical role in the regulation of the proximal *Nr3c1* promoter.

The binding of YY1 at the CGI shore region was further assessed in the PVN of control and ELS mice. While immunoblot analysis did not reveal changes in YY1 expression (Fig. 4F) *in vivo* ChIP studies showed region-specific reduction of YY1 occupancy at the CGI shore region in ELS vs. control mice (Fig. 4G), consistent with the observed hypermethylation of CpG3 in ELS animals (Fig. 2B) and the methylation-sensitive mode of YY1 binding to DNA (Fig. 3).

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. S8).

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 1₇ (rat), 1F (human) or the mouse orthologous region,^{9,16,49-53} in addition, some authors have reported changes in the expression of multiple GR transcript variants.^{15,23} Although a large proportion of the *Nr3c1* locus appears subject to epigenetic regulation,^{23,24} 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.³⁵ These residues localize more distal to the regions that are

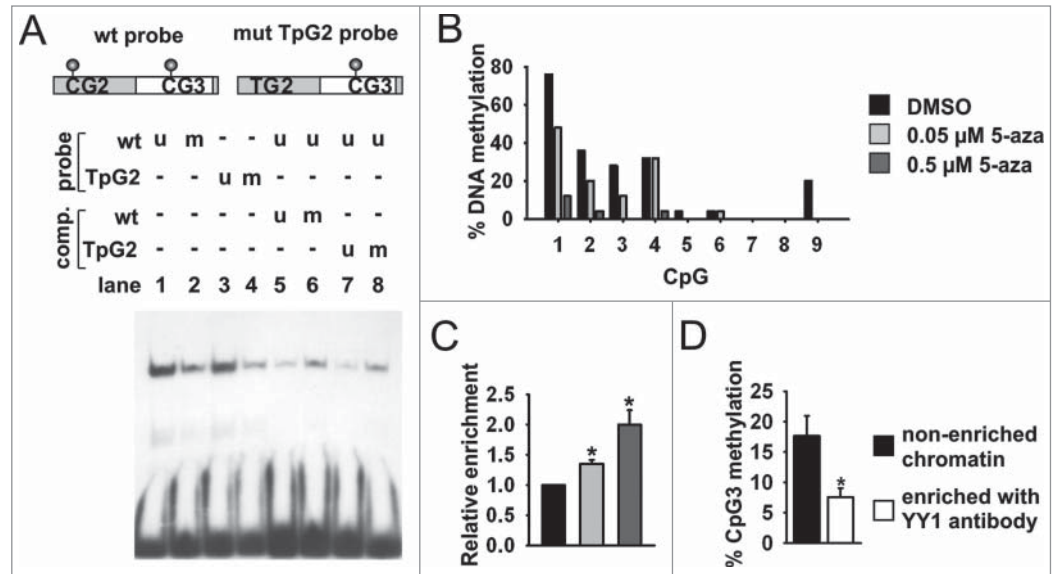


Figure 3. Methylation-sensitive YY1 binding to CGI shore. (A) Schematic view of GR wild type (wt) and CpG2 mutated probe (mut TpG2; upper panel). Representative EMSA shows methylation-sensitive binding of YY1 to *in vitro* methylated (m) CpG3 at the wt and mut TpG2 probe (lanes 1–4). YY1 binding to ³²P-labeled probes was competed with a fold5- molar excess of unmethylated and methylated wt and mut TpG2 unlabeled competitors (comp.) (lanes 5–8; lower panel). (B) Methylation levels of *GR* CGI shore region of N6 cells after treatment with increasing concentrations of 5-azacytidine (5-aza). (C) Relative values of binding of YY1 to CGI shore region by ChIP in N6 cells either treated with DMSO or increasing concentrations of 5-aza (**P* < 0.05 by one sample t-test; n = 4 experiments). (D) Methylation of non-enriched and YY1-immunoprecipitated (αYY1) chromatin was analyzed by single clone bisulfite sequencing (**P* < 0.05 by paired t-test; n = 3 ChIPs; PVN tissues from 2–3 mice were pooled for each ChIP). Data are means ± SEM.

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.³⁴

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 insulation-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),²⁷ *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)^{28,30} 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).²⁹ The

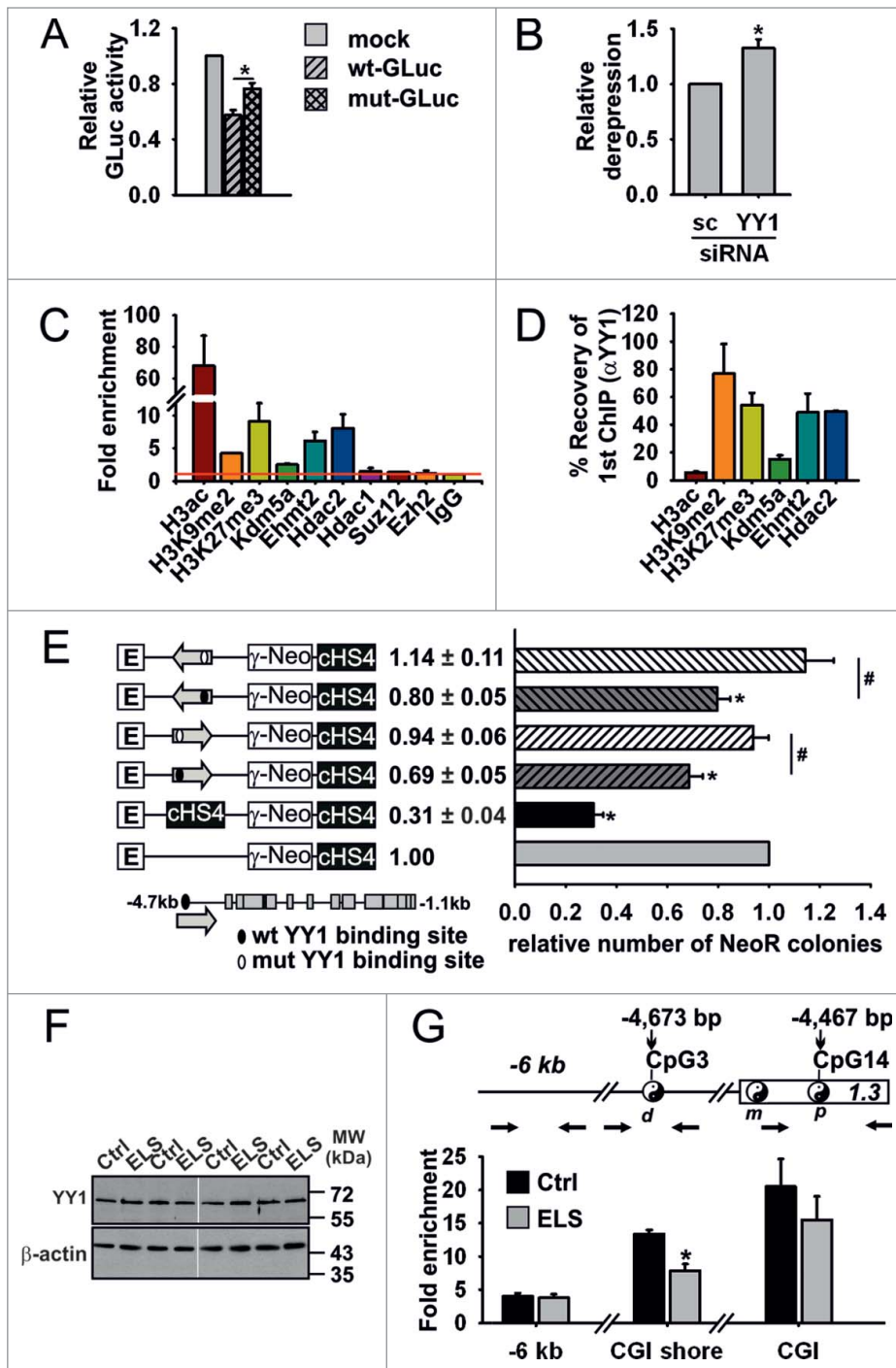


Figure 4. YY1 repressor binding is selectively reduced by ELS. **(A)** Relative values of repressive activity of the CGI shore region containing wt or a C-to-T mutation of CpG3 (mut) in N6 cells in a reporter assay (* $P = 0.01$ by paired t-test, $n = 4$ experiments) **(B)** Effect of YY1 and scramble (sc) siRNA on repressive activity of CGI shore region in N6 cells (* $P < 0.05$ by one sample t-test; $n = 4$ experiments). **(C)** Sequential ChIP analysis shows presence of H3ac, H3K9me2 and H3K27me3, Kdm5a, Ehmt2 and Hdac2 at the CGI shore after first ChIP step ($n = 3$ ChIPs; PVN tissues from 2–3 mice were pooled for each ChIP). **(D)** Subsequent ChIP with YY1-specific antiserum reveals enriched recovery of chromatin, which was precipitated with antibodies against H3K9me2 and H3K27me3, Kdm5a, Ehmt2, and Hdac2. **(E)** Insulation activity of *Nr3c1* CGI shore. The CGI shore region was inserted in both orientations between the γ -globin promoter (γ) and the β -globin enhancer HS2 (E) that drives expression of a neomycin resistance gene (Neo). Arrows indicate orientation and coverage of the region. The presence of insulator activity diminishes the effect of the enhancer on Neo expression and the formation of viable cell colonies in the presence of neomycin. An interposed core sequence of the chicken β -globin locus (cHS4) served as a positive control for efficient insulation. Insertion of the CGI shore sequence in either orientation reduced the number of viable colonies (* $P < 0.05$ by one sample t-test). Mutation of the YY1 binding site negated insulation, consistent with the idea that the CGI shore function is subject to regulation by YY1 ($\#P < 0.05$ by paired t-test, $n = 4$ experiments). **(F)** YY1 expression in the PVN of control and ELS mice. Representative immunoblot of 3 independent experiments. Molecular weight marker (kDa) indicated. **(G)** YY1 occupancy at the -6 kb region, the distal (d) YY1 binding site and the proximal (p) site of the GR promoter was analyzed in Ctrl and ELS mice by *in vivo* ChIP (ELS effect on region-specific binding; $P = 0.016$ by one-way MANOVA; * $P < 0.01$ by univariate F-test; $n = 5$ ChIPs per group; PVN tissues from 3 mice were pooled for each ChIP). Data are means \pm SEM.

products of all of these genes are implicated in stress-related affective disorders,^{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.^{2,55,56} This interesting observation suggests that ELS buffers against subsequent lifetime stressors, a phenomenon also recently reported in rodents and humans.⁵⁷⁻⁵⁹

Glucocorticoids inhibit hypothalamic synthesis and secretion of hypothalamic CRH, their actions being mediated by GR.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.⁴⁵ 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.⁶⁰⁻⁶²

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 1₇ in the hippocampus and enhanced binding of the transcriptional activator NGFI-A.⁹ In contrast, we show here that early-life adversity resulted in hypermethylation of *Nr3c1* at

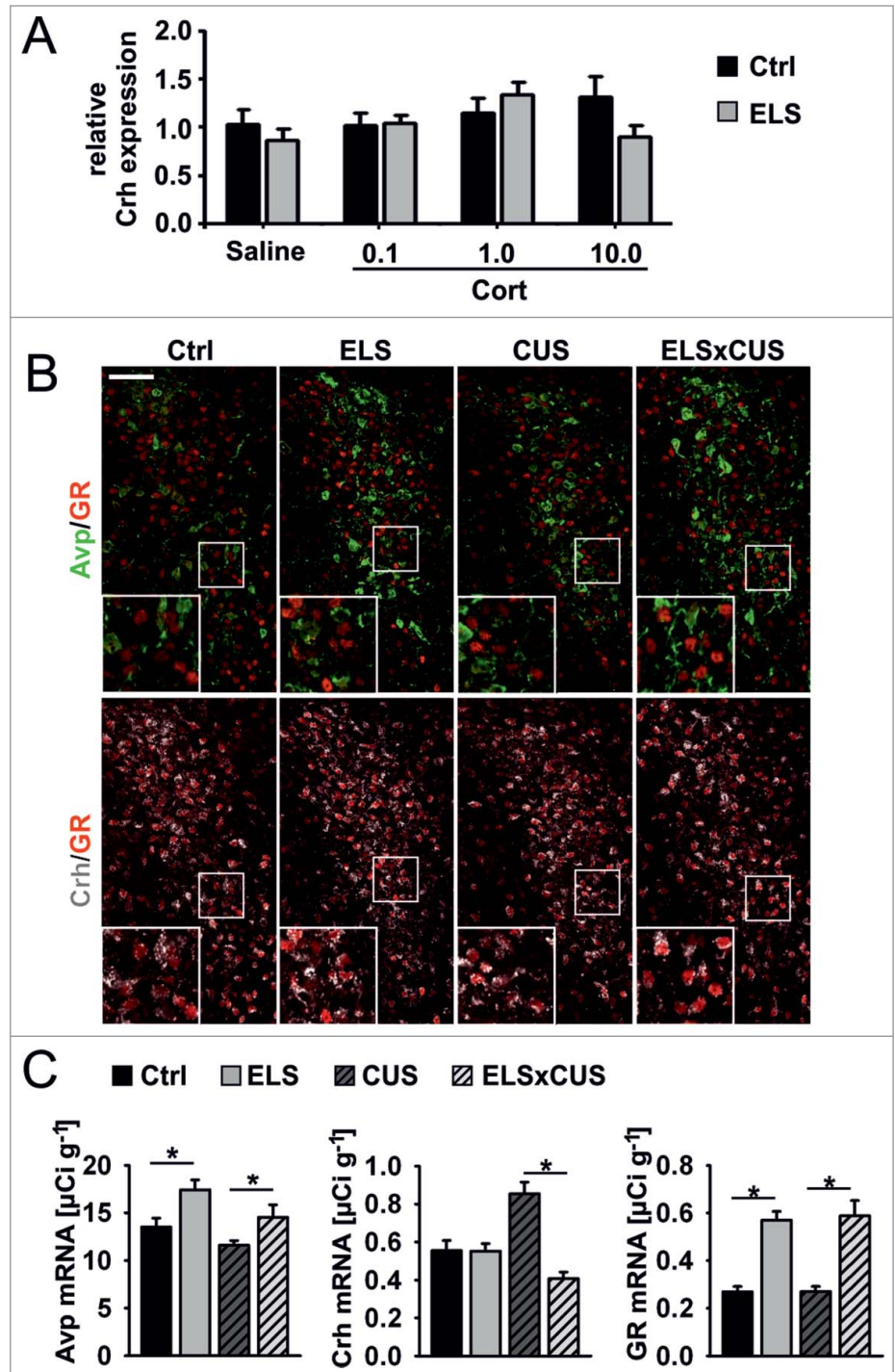


Figure 5. Glucocorticoids and chronic stress differentially regulate Crh expression. (A) Effect of intraperitoneally injected corticosterone (Cort; 0.1, 1.0, 10.0 mg kg⁻¹) on Crh expression in PVN of Ctrl and ELS mice as measured by qPCR. Expression data are normalized to *Atp5j*. (B) Representative immunohistochemical images showing GR, Crh and Avp staining on PVN sections from Ctrl, ELS, CUS and ELSxCUS mice. Scale bar = 50 μm. (C) Avp, Crh and GR mRNA detected by ISH (interaction of ELS and CUS; $P = 0.004$ by 2-way MANOVA; * $P \leq 0.001$ by univariate F-tests; $n = 7-15$). Data are means \pm SEM.

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.²⁵ A slightly modified version of a uCMS protocol was used.^{63,64} 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 × 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 × 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.²⁵ 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 $\Delta\Delta C_t$ method with efficiency correction as described.⁶⁵ Methylation analysis of genomic DNA (100–200 ng) was performed as described.²⁵ 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 *Bam*HI and *Nco*I 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 Gussia-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 CELLutions, Burlington, Canada), culture conditions and 5-azacytidine treatment were described elsewhere.²⁵ N6 cells (2×10^5) were transfected with 4 μ g plasmid DNA and 6 μ l 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.²⁵ 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 μ g GLuc expression vector and 5 μ l Lipofectamine 2000 (Life Technologies, Darmstadt, Germany). Sequences were: YY1 sense (5'-ucaggaggugaguucucuc-3') and antisense (5'-gagagaacucaccuccuga-3')⁶⁶ and scramble siRNA sense (5'-agguaguguaaucgcuug-3') and antisense (5'-caagggc-gauuacacuaccu-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 SssI (New England Biolabs, Frankfurt am Main, Germany) according to manufacturer's instructions. Unmethylated DNA was treated the same, but without SssI. Following phenol/chloroform extraction, completeness of methylation was controlled by digesting both methylated and unmethylated plasmids using methylation-sensitive restriction enzyme *Tau*I.

Electrophoretic mobility shift assays (EMSA)

Double stranded oligonucleotides were obtained by *Hind*III and *Nco*I 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). Electroporation of LLC-PK1 cells [ATCC CL-101] was used to efficiently express an YY1 expression vector (pCMV-YY1). About 4 μ g of nuclear extract was incubated alone or with an excess of competitor consensus oligonucleotide (yy1), its mutated form (yy2)⁶⁷ or with YY1- or pCAF-specific antibody under binding conditions described elsewhere.⁶⁸ Thereafter, 1 μ l 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 $2\text{--}3 \times 10^6$ N6 cells and PVN punches from 2- to 3-month old mice were performed as described elsewhere.²⁵ 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 saline-treated 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 cryopreserved. Cryostat sections (30 μ m) 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 Crh 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-YY1 or an equal amount of filling plasmid into LLC-PK1 cells.²⁵ 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 antiserum (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 *SalI* digest (0.5 µg) were electroporated into 5 × 10⁶ K562 cells [ATCC CCL-243]. pNI and pNI-CD plasmids⁴⁴ 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 methylthiazolyl-diphenyl-tetrazolium (MTT) after 2 weeks and counted. Each construct was tested in duplicate in 4 separate experiments using pooled plasmid DNA derived from 2 independent plasmid preparations.

In silico analysis, statistics

Emboss CpGplot (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/>) was used to identify CGIs.²⁵ Transcription Element Search

System (TESS) was used to predict the YY1 binding site covering CpG3 (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

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 (ANOVAs or MANOVAs) 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 artan-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 $\alpha = 0.05$ 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.

Acknowledgments

We gratefully acknowledge the support of A Varga and his team for help with animal care, A Yassouridis for statistical advice and analysis. We thank T Shenk (Princeton University) for providing us plasmids, pGEX2T-YY1 and pCMV-YY1, G Felsenfeld and A West (University of Glasgow) for the insulation vectors pNI and pNI-CD, and C Plank (Technical University of Munich) for K563 cells. We also thank H Gainer (NIH, Bethesda) for the Avp-specific antibody, PS 41.

Funding

This work was funded by the European Union Directorate General for Research & Innovation through the CRESCENDO Consortium (O.F.X.A. and D.S) and the NINA Initial Training Program (D.S. and O.F.X.A); the European Union did not influence the design, execution, or interpretation of the work described in this paper.

Supplemental Material

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

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