Methylation at the CpG island shore region upregulates \textit{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 (\textit{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 \textit{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 \textit{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.\textsuperscript{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).\textsuperscript{1,2}

Epigenetic mechanisms are increasingly recognized for their role in the dynamic transduction of the effects of changing environments on the genetic blueprint.\textsuperscript{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.\textsuperscript{4}

The glucocorticoid receptor gene (\textit{NR3C1}) encodes a ligand-gated transcriptional regulator that controls endocrine responses to stress as well as metabolism, inflammation, and reproduction.\textsuperscript{5}

The structure of \textit{NR3C1} is highly conserved between human, rat,\textsuperscript{6} and mouse\textsuperscript{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.\textsuperscript{8}

Pioneer studies in the rat showed that persistent changes in exon 1\textsubscript{7} DNA methylation occur as a function of quality of maternal care\textsuperscript{9}; subsequently, childhood trauma,\textsuperscript{10-14}
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). Comprehensive analysis of the NR3C1 gene in rats and human 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) 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. S1).

Nr3c1 contains multiple 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 5), 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. and encode a maximum of 2 glucocorticoid response elements (GREs). 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) 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. Also, no significant differences in DNA methylation of the mouse Nr3c1 proximal promoter (including the rat homolog of exon 1, 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.” Shore regions show a lower CpG content compared to canonical CGIs and frequently undergo DNA methylation. 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. 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. 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

248 Volume 10 Issue 3 Epigenetics

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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

248 Volume 10 Issue 3 Epigenetics
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 antisera specifically directed against YY1 was used (Fig. S5). Bisulfite sequencing of YY1-immunoprecipitated DNA recovered from the PVNs of ELS-naive 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. 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 YY1s 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

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**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 ≤ 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 ± 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 from upstream regulatory influences. Using a well-established proximal that the CGI shore region regulates transcription across the entire whether the CGI shore region shields might affect this function. We therefore next investigated 250 Volume 10 Issue 3Epigenetics

Figure 2. Methylation of proximal GR promoter and ELS-induced hypermethylation at CGI shore. (A) Map of the proximal untranscribed 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 ± SEM.

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 mice45 (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)

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 transcrption in vivo are barely affected by alterations of the corticosterone milieu over a broad range of concentrations.45

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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, 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 parvoellular 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 (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 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), 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).
products of all of these genes are implicated in stress-related affective disorders, 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. This interesting observation suggests that ELS buffers against subsequent lifetime stressors, a phenomenon also recently reported in rodents and humans.

Glucocorticoids inhibit hypothalamic synthesis and secretion of hypothalamic CRH, their actions being mediated by GR. Intriguingly, ELS and ELSxCUS mice showed control-like levels of 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, Emt2, and Hda2 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, Emt2, and Hda2. (E) Insulation activity of Nc3cr1 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 insulator 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 (HP < 0.01 by one sample 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 ± SEM.
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 17 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. A slightly modified version of a uCMS 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

**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 SsoI (New England Biolabs, Frankfurt am Main, Germany) according to manufacturer’s instructions. Unmethylated DNA was treated the same, but without SsoI. 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 Neuro 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 μg of nuclear extract was incubated alone or with an access of competitor consensus oligonucleotide (yy1), its mutated form (yy2) or 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 × 10^6 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 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 cryo- preserved. Cryostat sections (30 μm) were thawed, re-fixed in

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254 Volume 10 Issue 3 Epigenetics
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 antibodies, YY1-specific antisera, immunoblotting

**Disclosed 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.

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