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Glucocorticoid-induced loss of DNA methylation in non-neuronal cells and potential involvement of *DNMT1* in epigenetic regulation of *Fkbp5*

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Abstract

Glucocorticoids may play a significant role in the etiology of neuropsychiatric illnesses. Abnormalities in plasma cortisol levels, glucocorticoid sensitivity, and HPA-axis function often accompany clinical symptoms of stress-related illnesses such as PTSD and depression. Of particular interest are genetic association studies that link single nucleotide polymorphisms of HPA-axis genes with illnesses only in the context of an early-life trauma exposure such as child abuse. These studies suggest that dysregulation of HPA-axis function can have lasting repercussions in shaping mood and anxiety, long after termination of the traumatic experience. As *persistent* glucocorticoid-induced loss of DNA methylation in *Fkbp5* was previously observed in the hippocampus and blood and in the neuronal cell line HT-22, we asked whether these epigenetic alterations occur in non-neuronal, HPA-axis relevant cells. We used the pituitary adenoma cell line AtT-20 to demonstrate that the intronic enhancer region of *Fkbp5* undergoes loss of DNA methylation in response to dexamethasone treatment in a dose-dependent manner. We also focused on the mouse hippocampal dentate gyrus to test whether these changes would be enriched in a region implicated in the HPA-axis stress response, neurogenesis, and synaptic plasticity. We observed an increase in enrichment of DNA methylation loss in the dentate gyrus, as compared to whole hippocampal tissues that were similarly treated with glucocorticoids. We then asked whether *Dnmt1*, a methyltransferase enzyme involved in maintaining DNA methylation following cell division, is involved in the observed epigenetic alterations. We found a dose-dependent decrease of *Dnmt1* expression in the AtT-20 cells following dexamethasone treatment, and a similar decrease in corticosterone-treated mouse hippocampus. Taken together, we provide evidence that these glucocorticoid-induced epigenetic alterations have a broader validity in non-neuronal cells and that they may involve the DNA methylation machinery.

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Keywords

Fkbp5; *Dnmt1*; epigenetics; DNA methylation; AtT-20; pituitary; hippocampus; HPA-axis

1. Introduction

Glucocorticoids (GCs) may play a significant role in the etiology of several neuropsychiatric illnesses. A model for evaluating the role of GCs in mood disorders is Cushing's syndrome (CS), due to an endogenous increase in cortisol secretion, or secondary to iatrogenic GC treatment. Remarkably, up to 60~90% of patients with endogenous CS develop depression [1,2,3], and the depressive symptoms often disappear with resolution of hypercortisolemia [2,4]. In addition, non-CS patients suffering from major depressive disorder (MDD) often present with elevated levels of plasma cortisol and glucocorticoid resistance [5], further implicating glucocorticoids in mood disorder biology.

Effects of glucocorticoids are mediated in part by the glucocorticoid receptor (GR) and its associated chaperone protein complex consisting of FKBP5, HSP70, and HSP90. Upon activation by GC binding, GR dissociates from the chaperone complex, homodimerizes with other GR molecules, and translocates into the nucleus by interactions with FKBP4 and members of the dynein family of motor proteins [6]. In the nucleus, the GR dimer acts as a potent activator of transcription by binding glucocorticoid response elements (GREs) and recruiting transcription factors to gene promoters [7]. Importantly, FKBP5 is one of the immediate-early target genes of GC action, and GC induction of FKBP5 provides a short intracellular negative feedback loop, where FKBP5 reduces its own transcription by impeding further translocation of the GR complex. As a result, FKBP5 has been shown to be a strong modulator of GC-signaling *in vitro*, as levels of FKBP5 reduce GR's affinity for GCs in a dose-dependent manner [6]. This event, along with GC-induced changes in levels of GR [8,9], forms the molecular basis for glucocorticoid resistance.

An animal model of hypercortisolemia and GC resistance provides additional *in vivo* support for FKBP5 as a key mediator of GC sensitivity. GC resistance in squirrel monkeys, New World primates of the genus *Saimiri*, has been attributed to elevated levels of FKBP5, and transfection assays implicate species- and sequence-specific FKBP5 as the key modulator of GC resistance in this primate [10,11]. In humans, several single nucleotide polymorphisms (SNPs) in *FKBP5* have been associated with depression, altered HPA-axis function, and rapid response to antidepressants. MDD patients that carry these SNPs exhibit symptoms of GC resistance in that they often fail to properly suppress their endogenous cortisol levels following dexamethasone suppression test [12,13,14]. Specifically, SNP rs1360780 confers decreased sensitivity to GCs by causing elevated FKBP5 protein levels [12], consistent with symptoms of GC resistance often comorbid with MDD.

Recently, results from several candidate gene association studies have linked *FKBP5* to MDD, suicide, or PTSD, *only* in the context of previous stressors such as early-life trauma or child abuse events, and have begun to highlight gene-environment interactions as crucial factors for disease development [15,16,17,18,19]. These studies suggest that the HPA-axis may become dysregulated during early-life traumatic events and that this alteration persists through many years, even decades, to help precipitate mental illnesses later in life. These findings also suggest that long-term consequences of these early-life traumatic events may be potentiated by DNA sequence-independent, non-mutational events that chronically alter function of HPA-axis genes. To comprehend the long-term consequences of GC exposure on the HPA-axis and mood disorder biology, an epigenetic approach may be useful.

During our efforts to identify GC-induced epigenetic alterations on candidate HPA-axis genes, we made several discoveries in *Fkbp5*: (i) GC administration decreases brain and blood DNA methylation (DNAm) in the *Fkbp5* gene; (ii) DNAm alterations in *Fkbp5* are associated with behavioral deficits in an animal model of Cushing's syndrome; and (iii) there is a persistence of GC-induced DNAm change in both blood and brain for up to 4 weeks following GC withdrawal [20,21].

To address whether the observed loss of DNAm is confined to cells of lymphocytic and neuronal origins, we chose the murine AtT-20 anterior pituitary corticotroph cells [22], as these cells synthesize adrenocorticotrophic hormone (ACTH), and its transcription is suppressed by GCs [23]. We also tested this cell line for GC-induced changes in mRNA levels of DNA methyltransferase *Dnmt1*, hypothesizing that GCs would decrease *Dnmt1* expression.

2. Materials and methods

2.1 Animals

Four-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in a temperature- and humidity-controlled room. At five weeks of age, animals were given *ad libitum* access to solutions containing corticosterone (Sigma-Aldrich, St. Louis, MO; 100 µg/ml with 1% ethanol; "CORT" group) or 1% ethanol ("VEHICLE" group) in place of their normal drinking water, and this treatment continued for four weeks. Solutions were made fresh daily. On the morning of the last day of treatment, tail blood was collected (~20 µl) and centrifuged to separate plasma. Plasma was frozen and stored at -80°C until assayed for corticosterone by radioimmunoassay (RIA). Following four weeks of treatment, the animals were euthanized, and their brains frozen on powdered dry ice and subsequently stored at -80°C. All procedures were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine and were performed in accordance with guidelines established in the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2 Brain dissections

Frozen brains were sectioned using a cryostat, and ~500 µm sections were mounted on plain glass slides. 19-gauge needles (0.686 mm inner diameter and 1.086 mm outer diameter) were used to punch out the dentate gyrus from sections containing the hippocampus (bregma -0.98 mm through -1.5 mm and bregma -1.5 mm through -2.0 mm) [24]. Dissected samples were stored at -80°C until processed for genomic DNA and mRNA.

2.3 Radioimmunoassay (RIA)

Plasma hormone levels were determined by commercially available RIA kits for corticosterone (MP Biomedicals, Costa Mesa, CA), according to manufacturer's instructions as previously described [25]. All samples were run in duplicate and comparisons were made within assay with a coefficient of variance of 3.9%.

2.4 Cell line

AtT-20 cell line (Atcc.org, Manassas, VA) derived from a mouse pituitary tumor was cultured using DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) under standard conditions (5% CO₂, 37°C). Cells were trypsinized and replated in six-well plates before treatment with dexamethasone (DEX; Sigma). Control samples were treated with an equal volume of EtOH as those treated with 1 µM DEX. Respective media were changed daily. Cells were split on the third day of

treatment to maintain them in log phase of growth. After 5 days of treatment, cells were harvested for genomic DNA, and mRNA.

2.5 Gene expression

Messenger RNA from hippocampus and AtT-20 cell line was obtained using the RNeasy Lipid Tissue Mini (Qiagen, Valencia, CA). QuantiTect Reverse Transcription Kit (Qiagen) was used to generate cDNA for quantitative real-time PCR. Negative reverse transcriptase samples were used to ensure the absence of contaminating DNA. All reactions were carried out in triplicate using 1X Taqman master mix (Applied Biosystems, Foster City, CA), 1X Taqman probes for each gene [*Fkbp5*, *Dnmt1*, and *Actb* (β -actin)], and 30 ng of cDNA in a total volume of 20 μ l. Real-time reactions were performed on an Applied Biosystems 7900HT fast real-time PCR system with standard PCR conditions (50°C for 2 min; 95°C for 10 min; and 60°C for 1 min for 40 cycles). To determine relative expression values, the $-\Delta\Delta C_t$ method (Applied Biosystems) was used, where triplicate threshold cycle (C_t) values for each sample were averaged and subtracted from those derived from the housekeeping gene *Actb*. The C_t difference for a calibrator sample was subtracted from those of the test samples, and the resulting $-\Delta\Delta C_t$ values were raised to a power of two to determine normalized relative expression.

2.6 DNA extraction and bisulfite conversion

Genomic DNA (gDNA) from mouse brain tissues and cell line was isolated with the Masterpure DNA Purification Kit, according to manufacturer's instructions (Epicentre Biotechnologies, Madison, WI). Concentration of the gDNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Rockford, IL), and 500 ng of the DNA was used for bisulfite conversion according to manufacturer's protocol (EZ DNA Methylation Gold Kit; Zymo Research, Irvine, CA).

2.7 Bisulfite PCR and pyrosequencing

We measured DNAm by pyrosequencing of the PCR products, which measures methylation variation at >90% precision [26]. Two sets of primers (outside: Forward 5'-GAAAAGTTTTTGAGAATTAAGTTTAT-3' and Reverse 5'-ATAACAAAACACCAAAAACCTCTA-3'; and nested: Forward 5'-TTGTTGTGGGTATGTATTGATGTT-3' and Reverse 5'-CTCTCTCAACAATATAACTATAAA-3') were designed to ultimately amplify a 206 bp intron 1 region of mouse *Fkbp5*. Another set of primers (outside: Forward 5'-GATGATTAGTTTTTTTAGTAGTGATGT-3' and Reverse 5'-CTTATTATTCTCTTACTACCCTAA-3'; and nested: Forward 5'-TAGTTTTTGGGGAAGAGTGTAGAGTTAT-3' and Reverse 5'-ATTTTAAAAACACAAAACACCCTATT-3') were designed to amplify a 307 bp intron 5 region of mouse *Fkbp5*. 25 ng of bisulfite treated DNA was used for each PCR reaction. An additional nested PCR was performed with 2 μ L of the outside PCR reaction and one biotinylated primer (other primer being unmodified). Amplification for both PCR steps consisted of 40 cycles (94°C for 1 min, 53°C for 30 sec, 72°C for 1 min). PCR products were confirmed on agarose gels. Pyro Gold reagents were used to prepare samples for pyrosequencing according to manufacturer's instructions (Qiagen). Percentage of methylation at each CpG as determined by pyrosequencing was compared between DNA from CORT- and VEHICLE-treated brain tissues, or DEX- and VEHICLE-treated cell line samples.

2.8 Statistical Analysis

DNAm and relative gene expression measurements were analyzed by t-tests to compare CORT-treated and VEHICLE-treated groups (Microsoft Excel). A p -value < 0.05 was considered statistically significant.

3. Results

3.1 Glucocorticoid-induced changes in *Fkbp5* gene expression and DNA methylation in cell lines

We first attempted to establish whether the previously observed glucocorticoid-induced changes in gene expression and DNAm in brain and lymphocyte could also be observed in the AtT-20 cell line. Several concentrations of DEX (0, 10, 50, 100, 500, and 1000 nM) were used to treat the AtT-20 cells for five days. We observed a steady dose-dependent increase in relative *Fkbp5* mRNA expression (Fig. 1A). The highest expression occurred at 500 nM concentration of DEX, with a 257% ($p = 0.004$) increase over controls, suggesting that 500 nM may have saturated the glucocorticoid-signaling system. We then assessed the gDNA for any changes in DNAm in the two *Fkbp5* intronic regions previously characterized as glucocorticoid response elements (GREs, Suppl. Fig. 1) [27]. Using bisulfite pyrosequencing, we found subtle but significant dose-dependent decreases in DNAm at all four CpGs flanking the intron 5 GRE (Fig. 1B). The largest reduction was found at the second CpG position at 500 nM DEX, where we observed a 17.3% decrease in DNAm ($p = 0.002$). No further reduction was observed at 1000 nM DEX concentration (15.8%, $p = 0.01$), further supporting our speculation that GR-signaling has become saturated at 500 nM. In contrast, we failed to observe any significant differences between control and treated cells at any of the doses of DEX when we assessed for DNAm in the intron 1 GRE (Fig. 1C).

3.2 Corticosterone-induced changes in hippocampal *Fkbp5* intronic DNA methylation

We have previously observed corticosterone-induced loss of *Fkbp5* DNA methylation in the whole hippocampus and hypothalamus. This time, we asked whether the epigenetic activity that we have also observed in AtT-20 cells would be enriched in the dentate gyrus, a region of the hippocampus that has been implicated in stress-response, neurogenesis, and antidepressant response [28,29,30]. Further, we were interested in whether focusing on a specific sub-region would allow us to observe glucocorticoid-induced DNAm differences in the intron 1 GRE. Mice were either treated with 1% EtOH vehicle-solution ("VEHICLE"; $N=12$) or 100 $\mu\text{g/mL}$ of corticosterone in their drinking water ("CORT"; $N=12$) for four weeks. At the end of the treatment period, plasma corticosterone levels were 454.7 ± 78.3 ng/mL in corticosterone-treated animals and 22.9 ± 6.0 ng/mL in the vehicle-treated animals. We performed bisulfite pyrosequencing following gDNA extraction from the hippocampal punches. At intron 5, we observed a significant 14.0% decrease in DNAm ($p = 4.5 \times 10^{-6}$) at CpG position 4, and more subtle but still significant DNAm differences at CpG-1 (5.7%, $p = 0.03$), CpG-2 (2.9%, $p = 0.001$), and CpG-3 (7.6%, $p = 9.3 \times 10^{-5}$) (Fig. 2A). As in the AtT-20 cell line, we did not observe any significant differences in DNAm between VEHICLE-treated and CORT-treated mice at the intron 1 GRE, which suggested site-specific methylation changes both *in vitro* and *in vivo* (Fig. 2B).

3.3 Decrease in *Dnmt1* expression in the AtT-20 cell line and mouse hippocampus

Given the crucial role played by DNA methyltransferase enzymes in maintaining status quo of DNAm in the cells, we asked whether the observed loss of DNAm could be explained by glucocorticoid-induced suppression of *Dnmt1* expression. We observed a dose-dependent decrease in *Dnmt1* expression with a maximum of 36.7% decrease ($p = 0.0002$) at 500 nM DEX concentration in AtT-20 cells. Interestingly, we observed no further decrease at the 1000 nM concentration, further supporting our previous observation with *Fkbp5* expression

that GR-signaling may have become saturated at 500 nM DEX concentration (Fig. 3A). We also assessed for *Dnmt1* expression changes in the hippocampal tissue of mice treated with corticosterone. We found a 14.1% decrease in *Dnmt1* expression ($p = 0.02$), suggesting a similar effect of glucocorticoids in the brain as in the cell line (Fig. 3B).

4. Discussion

In this study, we explored two aspects of *FKBP5*'s role in mediating the stress-response, namely its glucocorticoid-induced DNAm loss in non-neuronal cells that participate in the HPA-axis system and a potential epigenetic mechanism that may underlie the observed loss in DNAm. We found GC-dependent transactivation and loss of DNAm of *Fkbp5* in the AtT-20 pituitary cell line that mirrored what were previously observed in other cells [20,21], suggesting a common mechanism of action. Our findings demonstrate that GC-induced loss of DNAm can be generalized to include such tissues and cells involved in the HPA-axis system.

Next, we examined whether focusing on a stress-relevant hippocampal region such as the dentate gyrus would allow us to observe a larger magnitude of epigenetic changes compared to bulk hippocampal tissues. In three of the four intron 5 CpGs tested, we observed a greater decrease in DNAm in this region compared to bulk hippocampal tissue. This phenomenon may be due to a larger ratio of granule neurons vs. glial cells that populate this region of the hippocampus, as we also observed greater corticosterone-induced DNAm changes in a homogenous neuronal cell line [20]. Recently, fluorescence activated cell sorting (FACS) was employed by Iwamoto et al. [31] to demonstrate that DNAm patterns from neurons were distinct from those of non-neurons and bulk tissues. In our experiments, we found that selective dissection of hippocampal tissue that enriched for granule neurons of the dentate gyrus preferentially represented the DNAm change that we initially observed in the AtT-20 cells. Since this DNAm change is shared by both granule neurons of the hippocampus and the pituitary cell line, additional experiments are needed to identify the common mechanism that governs this activity.

In an effort to identify one of the potential modulators of GC-dependent loss of DNAm, we assayed both the cell line and the dentate gyrus for expression levels of *Dnmt1*. We found that GCs caused a dose-dependent decrease in *Dnmt1*, providing a potential mechanism for the observed loss of DNAm. There is emerging evidence that active demethylation of 5-methylcytosines (5-mC) involves the formation of an intermediate 5-hydroxymethylcytosine (5-hmC), followed by a base-excision repair process that exchanges the 5-hmC with cytosine [32,33]. On the other hand, there's also evidence that nuclear hormone receptors employ a different mechanism of demethylation, particularly one involving methyl-CpG binding domain 4 protein MBD4, and DNA methyltransferases DNMT1 and DNMT3B [34]. Since *Dnmt3b* is a *de novo* methyltransferase, and therefore unlikely to play a role in regions that are already methylated, we focused on the maintenance methyltransferase *Dnmt1* that methylates newly synthesized daughter strands following DNA replication. Given that cell lines and the dentate gyrus are capable of active cell proliferation [28,29], we propose a passive demethylation process that is mediated by reduction in *Dnmt1* expression coupled with cell replication that results in a failure to methylate the newly synthesized strand. It remains to be determined whether reduction in *Dnmt1* levels translates to global reduction of DNAm, and whether restoring its levels can rescue the loss of DNAm. Further, site-specific loss of DNAm in the intron 5 GRE, but not in the intron 1 GRE, suggests an interaction between the *GR* and *DNMT1* that may include mutually exclusive occupancy of the intron 5 GRE. Carefully designed chromatin immunoprecipitation (ChIP) experiments are necessary to confirm this relationship.

In this study, we have broadened the validity of GC-induced DNAm loss to include pituitary cells and have identified a potential epigenetic mediator of the stress response. A clearer understanding of GC target tissues and mechanism of gene function alterations are essential for the development of better therapeutic strategies for treatment of mood disorders and stress exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and acronyms

Fkbp5	FK506 binding protein 5
DNAm	DNA methylation
Dnmt1	DNA methyltransferase 1
HPA-axis	hypothalamic-pituitary-adrenal axis
CS	Cushing's syndrome
GC	glucocorticoid
DEX	dexamethasone

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Highlights

- Dose dependent increase in *Fkbp5* and decrease in *Dnmt1* expression by dexamethasone
- Site-specific loss of methylation of *Fkbp5* in pituitary cell line and hippocampus
- Glucocorticoid-induced epigenetic activity in hippocampus enriched in dentate gyrus

Fig. 1. Glucocorticoid-induced increase in *Fkbp5* gene expression and decrease in DNAm in the AtT-20 cell line. (A) *Fkbp5* gene expression increases with dexamethasone (DEX) treatment in a dose-dependent manner in the AtT-20 cell line. (B) Increase in gene expression was accompanied by statistically significant dose-dependent loss of DNAm of four CpG dinucleotides flanking the intron 5 GRE (glucocorticoid response element). (C) No dose-dependent change in DNAm was observed in the intron 1 GRE. Data are shown as mean $\pm$ SEM. * $p \leq 0.05$: compared to 0 nM DEX-treated samples.

Fig. 2.

Corticosterone-induced changes in hippocampal *Fkbp5* intronic DNAm (A) Loss of DNAm was found in the intron 5 GRE in the hippocampal tissue of mice treated with corticosterone ("CORT"; N=12), compared to vehicle-solution treated controls ("VEHICLE"; N=12). (B) No differences were observed in the intron 1 GRE. Data are shown as mean $\pm$ SEM. ** $p \leq 0.001$, * $p \leq 0.05$

Fig. 3. Glucocorticoid-induced decrease in *Dnmt1* gene expression in the AtT-20 cell line and mouse hippocampus. (A) *Dnmt1* gene expression decreased with DEX treatment in a dose-dependent manner in the AtT-20 cell line. * $p \leq 0.05$: compared to 0 nM DEX-treated samples. (B) Statistically significant decrease was also observed in the hippocampal tissue of mice treated with corticosterone (“CORT”; N=12). Data are shown as mean $\pm$ SEM. * $p < 0.05$.