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Alterations in DNA Methylation of *Fkbp5* as a Determinant of Blood-brain Correlation of Glucocorticoid Exposure

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Summary

Background—Epigenetic studies that utilize peripheral tissues to identify molecular substrates of neuropsychiatric disorders rely on the assumption that disease-relevant, cellular alterations that occur in the brain are mirrored and detectable in peripheral tissues such as blood. We sought to test this assumption by using a mouse model of Cushing's disease and asking whether epigenetic changes induced by glucocorticoids can be correlated between these tissue types.

Methods—Mice were treated with different doses of glucocorticoids in their drinking water for four weeks to assess gene expression and DNA methylation (DNAm) changes in the stress response gene *Fkbp5*.

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Contributors

RSL designed the study and wrote the manuscript. ERE performed most of the experiments (pyrosequencing and radioimmunoassays) and co-wrote the manuscript. GSW helped with interpretation of data. FS and PZ performed and oversaw the statistical analysis of results. XY performed the realtime PCR and some of the pyrosequencing. KLT provided her expertise in animal husbandry and sample collection. JBP provided valuable clinical insights and edited the manuscript. All authors contributed to and have approved the final manuscript.

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Results—Significant linear relationships were observed between DNAm and four-week mean plasma corticosterone levels for both blood ($R^2 = 0.68$, $P = 7.1 \times 10^{-10}$) and brain ($R^2 = 0.33$, $P = 0.001$). Further, degree of methylation change in blood correlated significantly with both methylation ($R^2 = 0.49$, $P = 2.7 \times 10^{-5}$) and expression ($R^2 = 0.43$, $P = 3.5 \times 10^{-5}$) changes in hippocampus, with the notable observation that methylation changes occurred at different intronic regions between blood and brain tissues.

Conclusion—Although our findings are limited to several intronic CpGs in a single gene, our results demonstrate that DNA from blood can be used to assess dynamic, glucocorticoid-induced changes occurring in the brain. However, for such correlation analyses to be effective, tissue-specific locations of these epigenetic changes may need to be considered when investigating brain-relevant changes in peripheral tissues.

Keywords

Fkbp5; Epigenetics; DNA methylation; Blood-brain correlation; Biomarker; and Glucocorticoids

1. Introduction

Levels of the glucocorticoid cortisol constitute one of the key determinants of allostasis or allostatic load, and prolonged exposure to its catabolic properties leads to numerous diseases that include diabetes, cardiovascular disease, and obesity (Karamanoglu et al., 2002). For the brain, exposure to glucocorticoids is a robust risk factor for neuropsychiatric illnesses and cognitive decline, as numerous lines of evidence implicate exposure to stress or glucocorticoids in the etiology of several psychiatric disorders (McEwen and Gianaros, 2010, Fardet et al., 2012, Theall et al., 2012). In particular, hypercortisolemia or dysregulation of the hypothalamic-pituitary-adrenal-axis (HPA-axis) system is often associated with mood disorders (Gillespie and Nemeroff, 2005), and genetic association studies have linked single nucleotide polymorphisms in “HPA-axis genes” that govern the stress-response with post-traumatic stress disorder (PTSD), suicide, and depression (Binder et al., 2004, Binder et al., 2008, Roy et al., 2010, Roy et al., 2012, Sinclair et al., 2012). Large-scale epidemiological studies have also implicated iatrogenic glucocorticoid administration with psychiatric symptoms in hundreds of thousands of patients prescribed steroids for non-psychiatric diseases (Fardet et al., 2012). In addition, a more direct, causal relationship between glucocorticoid exposure and psychiatric illnesses can be found in Cushing’s disease, where endogenous or iatrogenic elevation in plasma glucocorticoid levels has led to depression in 60–90% of patients (Cohen, 1980, Flitsch et al., 2000). Notably, the psychiatric symptoms are often alleviated with resolution of hypercortisolemia, strongly suggesting involvement of glucocorticoids in depressive symptoms (Starkman et al., 1986, Dorn et al., 1997). In animals, stress-induced deficits in both dopamine signaling and behavior are rescued by the glucocorticoid receptor antagonist mifepristone (RU486), further implicating glucocorticoid action (Niwa et al., 2013). Therefore, the ability to measure glucocorticoid exposure in the brain would serve as a useful gauge for assessing susceptibility to neuropsychiatric illnesses.

Previously, we found that degree of change in DNA methylation (DNAm) at a key regulatory locus within the stress-response gene *Fkbp5* robustly reflected the previous 30-

day mean plasma glucocorticoid values in the mouse blood (Lee et al., 2011). In fact, methylation changes in blood also correlated with anxiety-like behavior on the elevated plus maze. In addition, these epigenetic changes strongly correlated with functionally relevant physiological changes in glucocorticoid target tissues, such as atrophy of the thymus, spleen, and the adrenal glands. Thus we suggested in our previous study that using such an approach is highly practical since only one small sampling (~20 μ L of blood) accurately reflected and obviated the need for multiple daily measurements of plasma glucocorticoid levels when determining glucocorticoid burden (Lee et al., 2011).

In this study, we extend our analysis to the hippocampus, a particularly glucocorticoid-sensitive brain region (Kaouane et al., 2012), to determine whether the correlations between mean glucocorticoid exposure over a four-week treatment period and changes in DNAm and expression levels of the *Fkbp5* gene in the blood accurately reflect changes in the brain. In addition to its function as a stress-response gene and a regulator of glucocorticoid signaling (Scammell et al., 2001, Wochnik et al., 2005), FKBP5 has been implicated in several candidate gene association studies of depression, bipolar disorder, and PTSD (Binder et al., 2004, Binder et al., 2008, Willour et al., 2009, Roy et al., 2012). A recent clinical study implicating methylation changes of *FKBP5* in blood to childhood trauma exposure (Klengel et al., 2013) further warrants a closer examination of the relationship between blood and brain epigenetic changes in this important gene.

Given the link between glucocorticoid exposure and neuropsychiatric disorders, development of a tool that can assess exposure in the brain through the use of a surrogate, easily accessible tissue would be of great clinical utility. While reported as a case study of a single but an important gene in stress response, our results provide useful insights into the use of surrogate tissues for brain and suggest guidelines for future biomarker discoveries.

2. Materials and methods

2.1. Animals

At five weeks of age, male C57BL/6J mice (N=12 in each group; Jackson Laboratories, Bar Harbor, ME) were given *ad libitum* access to solutions containing the rodent stress glucocorticoid corticosterone (Sigma-Aldrich, St. Louis, MO; 100 μ g/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. This group is referred to as the first cohort. A second cohort of animals (N = 8 in each group, 5 dose groups, and 40 total) was treated with five doses of corticosterone (0, 25, 50, 75, or 100 μ g/ml with 1% ethanol), once again with *ad libitum* access to the solution in place of their drinking water, in order to obtain a wider dose-effect relationship. At the end of the treatment period, animals were euthanized, tissues harvested, 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. Blood collection

20 μ L of whole blood was collected weekly (0900 hr) from each mouse into heparinized glass capillary tubes through a small nick at the tip of the tail. Blood samples were centrifuged at 4°C and plasma was collected and frozen at –80°C for further analysis of total (free and bound) plasma CORT by radioimmunoassay, according to the manufacturer's instructions (MP Biomedicals, Costa Mesa, CA). All samples were run in duplicates with an intra-assay coefficient of variance (CV) of 3.9% and inter-assay CV of 7.5%. Animals were euthanized at the end of the treatment period, and trunk blood (~250 μ L) was collected. Blood samples were incubated with three-volumes of ACK Lysing Buffer (Quality Biological, Gaithersburg, MD) to lyse red blood cells, centrifuged to collect white blood cells, and divided into two fractions for subsequent genomic DNA (gDNA) and (mRNA) messenger RNA analysis.

2.3. Brain dissection

Frozen mouse brains were sectioned using a cryostat, and 400 μ m or 500 μ m sections were mounted on glass slides. 19-gauge needles (0.686 mm inner diameter and 1.086 mm outer diameter) were used to punch out the hippocampal dentate gyrus (bregma –0.98 through –2.0 mm). Punched tissues were stored at –80°C in either Tissue & Cell Lysis Buffer (Epicentre Biotechnologies, Madison, WI) or Qiazol (Qiagen, Valencia, CA) until processed for gDNA and mRNA analysis, respectively.

2.4. Messenger RNA extraction and gene expression

Messenger RNA from white blood cells and the hippocampal dentate gyrus was obtained using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). QuantiTect Reverse Transcription Kit (Qiagen) was used to generate cDNA for quantitative real-time PCR. Calculation of expression values has been previously described (Lee et al., 2011).

2.5. Genomic DNA extraction and bisulfite conversion

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

2.6. Bisulfite PCR and pyrosequencing

DNA methylation (DNAm) status was measured by pyrosequencing of the PCR products, which measures methylation variation at >90% precision (Colella et al., 2003). Procedures for PCR of bisulfite-converted DNA for pyrosequencing, including primer sequences, have been described previously (Lee et al., 2010). Percentage of methylation at each CpG (seven total: three CpGs from intron 1 and four CpGs from intron 5) as determined by pyrosequencing was compared between DNA from 0 μ g/mL (or VEHICLE) vs. all other CORT doses for both cohorts. Based on the UCSC Genome Browser mouse build mm10, genomic coordinates for the CpGs examined are: intron 1 CpG-1: 28,441,243; CpG-2:

28,441,253; CpG-3: 28,441,288; and intron 5 CpG-1: 28,420,359; CpG-2: 28,420,385; CpG-3: 28,420,541; CpG-4: 28,420,548, which are all located on chromosome 17.

2.7. Statistical Analysis

For the first cohort, comparisons of mean plasma CORT, tissue-specific DNAm and gene expression between CORT- and VEHICLE-treated animals were analyzed by Student's *t*-tests. In the second cohort, we used linear regression to assess the relationships of CORT exposure (captured as an ordinal variable for doses of 0, 25, 50, 75 and 100 µg/mL) with mean plasma CORT levels, mean plasma CORT levels with tissue specific DNAm and gene expression, and DNAm of specific CpGs in blood with DNAm of CpGs in the hippocampus. We computed R^2 and *p*-values from the linear regression models. *P*-values were adjusted by Bonferroni correction for multiple testing by the number of CpGs tested for each tissue. For instance, where all blood CpG DNAm values were compared to all hippocampal CpG DNAm values, corrected $p < 0.05$ [or uncorrected p -value $\times (7 \text{ blood CpGs} \times 7 \text{ hippocampal CpGs})$] was considered study-wide significant. All analyses were performed using R statistical programming (R, 2010).

3. Results

3.1. Glucocorticoid exposure and changes in *Fkbp5* expression

Genomic structure of the stress-response gene *Fkbp5* (Fig. 1a) includes two promoter CpG islands and two bioinformatically-annotated and functionally-characterized glucocorticoid response elements (GREs) that reside in the first and fifth introns. We first assessed the functional and epigenetic consequences of glucocorticoid exposure on the *Fkbp5* locus. A (first) cohort of mice were treated with 100 µg/mL of corticosterone (CORT) in the drinking water for four weeks. To adequately capture glucocorticoid exposure, plasma samples were obtained at weekly intervals. In the treated group, plasma CORT levels (mean $\pm$ SEM) became significantly elevated following the first week of treatment and remained elevated throughout the treatment period. In contrast, plasma CORT levels in the vehicle-treated animals remained less than 40 ng/mL during all sampling times (Fig. 1b, **left panel**; $p < 0.01$). The mean plasma CORT levels over the four weeks of treatment was 309.7 ± 27.0 ng/mL for treated animals (CORT, $N=12$) versus 28.2 ± 3.2 ng/mL for vehicle-treated animals (VEHICLE, $N=12$; $p = 2.1 \times 10^{-7}$, Fig. 1b, **right panel**). An additional repeated measures ANOVA test to assess the impact of chronic CORT administration on plasma CORT levels showed that four-week CORT administration had a significant effect on the difference in measured plasma CORT levels between exposed (CORT) and unexposed (VEHICLE) groups ($p = 2.0 \times 10^{-7}$).

In order to examine the impact of elevated CORT levels on *Fkbp5* gene function, mRNA was extracted from blood lymphocytes and hippocampus at the end of the treatment period and assayed for expression levels. We observed greater than four-fold increase in blood *Fkbp5* ($p < 0.001$) and a more modest ~80% increase in hippocampal *Fkbp5* ($p < 1.7 \times 10^{-3}$) in the CORT vs. VEHICLE animals (Fig. 1c). As *Fkbp5* is an immediate-early stress-response gene, our results further confirm robust CORT-induced increase in its expression levels in both tissues examined.

3.2. Glucocorticoid exposure and changes in DNA methylation

We then examined whether increases in expression levels observed in these tissues are accompanied by epigenetic alterations. In order to maximize observed epigenetic changes in the hippocampus, we carried out hole-punch dissection of the granule neuron-rich dentate gyrus. gDNA from white blood cells and the dentate was then bisulfite-treated and subjected to pyrosequencing. Two intronic regions previously characterized as glucocorticoid response elements (GREs) were assayed for changes in DNAm levels (Magee et al., 2006). The GRE in intron 1 includes three CpGs, whereas the GRE of intron 5 includes four CpGs. In blood, CpGs in intron 1 GRE showed significant changes in DNAm in CORT-treated animals, with notable changes in CpG-1 (36.7%, $p = 3.7 \times 10^{-17}$) and CpG-2 (34.4%, $p = 3.1 \times 10^{-15}$), and a more modest change in CpG-3 (11.6%, $p = 6.6 \times 10^{-14}$, Fig. 2a). In contrast, the intron 5 GRE in blood showed relatively small DNAm changes, with the magnitude of DNAm differences less than 6.5% at all four CpGs (Fig. 2b). A small but statistically significant DNAm change at CpG-4 (5.5%, $p = 0.003$) showed an increase in methylation that was inconsistent with the other CpGs in intron 5 and did not replicate in the second cohort of animals.

In the hippocampus, we observed small but significant differences in CORT-induced DNAm changes in intron 1 CpG-1 (2.7%, $p = 6.0 \times 10^{-5}$, Fig. 2c), which we deemed biologically irrelevant due to the small effect size. In contrast, all four CpGs in intron 5 in the dentate underwent significant DNAm changes with CORT treatment. The most remarkable epigenetic changes were observed at CpG-4, where the magnitude of methylation differences was 14.0% ($p = 1.8 \times 10^{-5}$, Fig. 2d). Pyrosequencing performed on other potential regulatory cis elements, including few CpGs within two of the three CpG islands and 10 CpGs of the promoter, were hypomethylated and did not show appreciable differences in DNAm (data not shown) (Lee et al., 2010).

3.3. Dose-response changes in plasma corticosterone levels, expression, and DNA methylation

In order to determine whether there were dose-response relationships between CORT exposure and changes in plasma CORT levels, gene expression, and DNAm, we treated another (second) cohort of mice with different doses of CORT (0, 25, 50, 75, and 100 $\mu\text{g}/\text{mL}$). A trend analysis revealed that mean plasma CORT levels were linearly related to increasing doses of CORT administration ($R^2 = 0.74$, $p = 1.18 \times 10^{-12}$). In turn, there were significant linear relationships between mean plasma CORT levels and epigenetic and expression changes in *Fkbp5* in both blood and brain tissue. In general, the most significant correlations between mean plasma CORT levels and DNAm were observed with the intron 1 CpGs in blood whereas they were with the intron 5 CpGs in the hippocampus (Table 1). In particular, a strong correlation was observed with intron 1 CpG-2 in blood ($R^2 = 0.68$, $p = 7.1 \times 10^{-10}$, Fig. 3a), which replicated our previous work (Lee et al., 2011). On the other hand, the most significant correlation was observed with intron 5 CpG-4 in the hippocampus ($R^2 = 0.33$, $p = 0.001$, Fig. 3b). There were also significant correlations between mean plasma CORT levels and expression of *Fkbp5*, but this was more apparent in the hippocampus ($R^2 = 0.37$, $p = 3.5 \times 10^{-5}$, Figure 3c) compared to blood ($R^2 = 0.14$, $p = 0.02$, Figure 3d).

3.4. Correlation between blood and brain DNA methylation

Finally, we examined the pairwise associations between DNAm values of all seven intronic CpGs in blood versus the hippocampus in this second cohort of mice (Suppl. Table). Taking the conventional approach of comparing the same CpGs across tissues, no significant correlations were observed ($R^2 = 0.24$ and $p = 0.064$, Suppl. Table). In contrast, more significant correlations were observed comparing different CpGs across introns. Comparisons between the three intron 1 CpGs in blood and the four intron 5 CpGs in hippocampus are shown in Table 2. The most significant correlation was observed between intron 1 CpG-2 DNAm in blood and intron 5 CpG-4 DNAm in the hippocampus ($R^2 = 0.49$ and $p = 2.7 \times 10^{-5}$, Fig. 4a). Interestingly, while expression of *Fkbp5* in the hippocampus was significantly correlated with intron 5 CpG-4 DNAm in the hippocampus ($R^2 = 0.29$ and $p = 0.003$, Fig. 4b), it was even more highly correlated with intron 1 CpG-2 DNAm ($R^2 = 0.43$ and $p = 3.5 \times 10^{-5}$, Fig. 4c). Taken together, these data suggest that glucocorticoid-induced methylation changes observed in blood gDNA can serve as a proxy to both methylation and expression changes in the brain.

4. Discussion

Many epigenetic studies that employ DNA from peripheral sources such as blood are predicated on the assumption that non-genetic alterations that have occurred in target CNS regions are mirrored in the periphery. This assumption is especially prevalent in epigenetic studies of neuropsychiatric disorders, where essential brain tissues are virtually inaccessible in patients, and investigators must rely on other sources of DNA, such as from blood draws (Mehta et al., 2013) or buccal swabs (Yang et al., 2013). However, there are only a few studies that have tested this assumption by capturing disease-relevant, dynamic changes in the brain and linking them to those occurring in peripheral tissues.

One such study examined the 1 γ promoter of the glucocorticoid receptor (GR) and reported that the sum of methylation values between whole blood cells and the prefrontal cortex of stressed and unstressed animals show moderate association on a Pearson correlation heat map (Witzmann et al., 2012). Another study has performed a methylomic profile of blood, cortex, and hippocampus and found relatively high degree of concordance of 62.3% in DNAm among the three tissues and similar concordance rates of methylation changes among tissues of mice that were treated with haloperidol (Aberg et al., 2013). Other interesting blood-correlation studies have identified numerous loci that have both similar and tissue-specific epigenetic patterns between blood and brain tissues of “control” individuals (Davies et al., 2012), and loci that maintain strong across-tissue concordance across age (Horvath et al., 2012). High across-tissue concordance rates provide support for using blood DNA as a proxy for that of brain tissues and the motivation to investigate specific genomic loci for underlying mechanisms in greater detail and under different conditions and treatments.

In this study, we sought to establish a correlative relationship between two tissues by measuring dose-dependent DNA methylation changes that occur following exposure to glucocorticoids. Our work on the *Fkbp5* gene was initiated out of a search, both in the literature and by pyrosequencing, for candidate loci that could account for the expression

changes observed in both blood and brain of CORT-treated mice. Intron 1 CpGs had been characterized as an important binding site for MECP2 (Nuber et al., 2005), was located adjacently to a potential GRE (Magee et al., 2006), and had shown substantial CORT-induced reduction of DNAm in blood (Lee et al., 2010). However, intron 1 CpGs were mostly unaffected in the hippocampus. Pyrosequencing of additional candidate regions revealed significant DNAm changes in a highly conserved GRE in intron 5, prompting us to ask whether any significant relationships could be derived between measurements made in blood and those made in hippocampus for glucocorticoid exposure.

Despite the presence of tissue-specific changes in DNAm, we were able to derive modest, yet significant relationships between blood DNAm versus hippocampal DNAm and expression. As DNAm changes in both tissues were significantly correlated to overall CORT-exposure for each animal, DNAm measurements made from a drop of blood could provide useful information about the impact of CORT exposure in the hippocampus, thereby supplanting requisite serial measurements to properly assess CORT exposure and direct measurements of DNAm in the hippocampus. Since DNAm changes in blood was also significantly correlated with expression changes of *Fkbp5* in the hippocampus, it may also be possible to assess sensitivity and resistance of this tissue to glucocorticoids (Scammell et al., 2001). Similar studies with additional “HPA-axis” genes, such as *Gr*, *Fkbp4*, and *Hsp90*, are necessary to provide a more complete picture of glucocorticoid signaling in the brain.

Our findings also raise an intriguing question regarding the nature of the epigenetic architecture at the *Fkbp5* locus. We observed very similar DNAm levels in the intronic regions of both blood and hippocampus. In *both* tissue types, two of the three CpGs of intron 1 are highly methylated at 80% DNAm, whereas intron 5 is relatively hypomethylated, with three of the four CpGs close to 20% DNAm. Why are the DNAm changes intron- and tissue-specific, despite the presence of similar methylation patterns at these two loci in both tissues? It is unclear to us at this time as to the different functional, regulatory modalities that may exist at these intronic regions between these tissues. It is possible that despite similar DNAm patterns, blood and hippocampus may harbor vastly dissimilar histone modification patterns. Histone modifications and chromatin remodeling have been investigated for intron 5 (Paakinaho et al., 2010), and it would be interesting to see whether similar or different histone marks exist in intron 1 for blood and brain tissues. Given this possibility, differential histone patterns, or differential chromatin conformation, may confer differential accessibility of the GR-complex to the intronic regions. Differential histone and GR occupancy may work in conjunction to affect gene expression during acute CORT exposure, and these may then interact with CORT/GR-mediated changes in DNAm to affect the timing and robustness of gene expression during chronic CORT exposure. A rigorous investigation of histone modifications and CORT-mediated occupancy of GR at these two introns is needed to adequately answer this question. While these additional mechanisms have the potential to confound current and future studies that compare across-tissue epigenetic marks, they also provide additional avenues to alter or mitigate the impact of excessive exposure to glucocorticoids.

Our findings highlight several other biological considerations. First, our ability to observe blood-brain correlation of CORT-exposure depended largely on the presence of functional

glucocorticoid signaling in both of the tissues examined. It was due to this fact that we were able to obtain dose-dependent DNAm changes at GREs that significantly reflected CORT exposure in both tissue types, and made it possible to correlate those changes between the two tissues. Further, expression changes caused by elevated glucocorticoid levels in the brain provided another parameter that was significantly associated with DNA methylation changes in the blood, and this was also due to active GR-signaling. It is unknown at this time whether loci that are transcriptionally active in only one of the two tissues both undergo similar epigenetic changes.

Second, tissue heterogeneity represented a significant confounding factor in assessing epigenetic changes. This issue was especially relevant in the brain where presence of astrocytes, glia, oligodendrocytes, and microglia vastly outnumber neurons, and any significant epigenetic changes that occur in one population of cells is likely to be diluted by static epigenetic values of others. In the current study, targeting specific sub-regions such as the granule neuron-rich dentate gyrus allowed us to observe significantly greater differences in glucocorticoid-induced DNAm changes that were not observed in a previous study (Lee et al., 2010). Based on our data and work by others, we suggest that fluorescence-activated cell sorting (FACS) of neurons may be a more effective way to enrich for DNAm differences between treated and control groups (Iwamoto et al., 2011). However, since FACS does not discriminate among subregions of the hippocampus such as the CA1, CA3, and dentate gyrus, we opted to hole-punch dissect the dentate. While white blood cell populations are also likely to be affected by tissue heterogeneity, we speculate that the large effect size observed in one or more tissue types has allowed us to derive a strong correlation of blood DNAm to mean CORT levels. Immunophenotyping can accurately identify and isolate subpopulations of white blood cells that undergo glucocorticoid-induced epigenetic changes. Recent epigenetic studies have begun to employ isolation techniques such as retrograde bead labeling coupled with FACS (Niwa et al., 2013) and laser capture microdissection (Hackler et al., 2012) to maximize the observable epigenetic changes. Additionally, bioinformatics approaches have enabled the development and use of novel statistical methods to account for cellular heterogeneity in brain (Guintivano et al., 2013) and blood tissues (Houseman et al., 2012).

Third, contrary to our initial expectations, DNAm changes in the current study did not occur in CpG islands or promoters, despite the fact that mouse *Fkbp5* gene possesses three CpG islands, one intronic and two promoter, that could have served as potential sites of glucocorticoid regulation and epigenetic alteration. In fact, emerging evidence in epigenomics demonstrates that much of tissue-specific, disease-relevant epigenetic changes occur within CpG island “shores,” or ~2.5kb regions that flank CpG islands (Irizarry et al., 2009). In our study, we found that glucocorticoid-induced DNAm changes occurred tens of thousands of kilobases away from the *Fkbp5* promoter and CpG islands. These targets of epigenetic modification were bioinformatically and/or experimentally characterized *intronic* GREs that have been demonstrated to bind the methylation-dependent repressor MECP2 or the activated GR complex (Nuber et al., 2005, Magee et al., 2006, Paakinaho et al., 2010). These findings necessitate a careful consideration in the design and implementation of target probes used in genome-wide DNAm microarray or bisulfite sequencing platforms. For

instance, additional regions such as DNase- hypersensitive sites that encompass binding of transcription regulators or cis-elements (Boyle et al., 2008), or regulatory regions as demonstrated by chromatin immunoprecipitation (ChIP) with specific transcription factors or histone modifications followed by next-generation sequencing (ChIP-Seq) (Rye et al., 2011), may need to be included in platform design to identify regions of active epigenetic alterations.

Our current study has several limitations. First, although observed levels of CORT may be relevant to endogenous or iatrogenic Cushing's disease, the exposure amount is likely excessive for modeling conditions of chronic stress exposure. Therefore, it is important to verify whether the findings from the present model are also identified in stress models. Second, it is not clear whether our findings can be translated to humans. While the intron 5 GRE is highly conserved in all of the rodent and primate species examined, the intron 1 GRE is limited to mice and rats (UCSC Genome Browser). Therefore, it is not clear whether patients exposed to high levels of glucocorticoids will exhibit blood DNAm changes in a homologous region in intron 1. For example, a recent study in human blood has reported lymphocyte-specific DNAm changes near the intron 2 GRE (Klengel et al., 2013), raising the possibility that cross-species GRE concordance may not be perfect. Lastly, it is possible that the observed tissue-specific epigenetic patterns may only apply to the *Fkbp5* gene. Until similar across-tissue DNAm changes are identified in other glucocorticoid-responsive genes, it is imprudent to make any generalizations regarding our observations. Specifically, it is not clear whether other genes that are important for mood disorders and have been demonstrated to be expressed in lymphocytes, such as *Crh*, *Gr*, and *Bdnf* (Baker et al., 2003, Zuccato et al., 2011, Maranville et al., 2013), exhibit similar across-tissue, CpG-to-CpG correlations. A methylomic assay, such as next-generation sequencing of bisulfite-converted target DNA, is warranted to establish whether the observed tissue-specific DNAm of *Fkbp5* is the norm or the exception, and to determine what other genes, pathways, and physiological processes are affected by exposure to glucocorticoids.

Despite these limitations, our study lends support to using surrogate tissue for the brain with the caveat that the particulars of epigenetics biology that determine across-tissue correlates be taken into consideration. A next step in this regard is to determine how much of the results from this preclinical model may apply to the human condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a) Genomic Organization of Mouse *Fkbp5*

b)

c) *Fkbp5* Expression in Blood and Hippocampus **Figure 1.**

Genomic organization of the mouse *Fkbp5* gene and effect of chronic glucocorticoid exposure on its expression and DNA methylation. (a) Mouse *Fkbp5* genomic locus (Chr.17: 28,399,095 – 28,517,524, UCSC Genome Browser, mm10) consists of eleven exons (black boxes), four alternate exons (gray boxes), and three CpG islands (thick horizontal lines), two of which are located upstream of the first alternate exon and the third one immediately

downstream of the first exon. Two GREs (glucocorticoid response elements) assayed by pyrosequencing for tissue-specific DNA methylation are represented by thin horizontal lines. **(b)** To assess the impact of chronic CORT exposure on *Fkbp5* gene expression and methylation, animals were treated for four weeks and serial, weekly measurements for plasma CORT levels were determined. For each group (VEHICLE vs. CORT), an average was calculated from individual animals at each of the weekly CORT measurements (left panel), and weekly mean values from each group were averaged further to obtain mean plasma CORT level differences between the two groups (right panel). **(c)** After four weeks of CORT treatment mRNA was extracted from blood and hippocampus. Tissues examined from the CORT-treated group showed elevation of *Fkbp5* expression, with blood showing the greater increase. Data are shown as mean $\pm$ SEM. ** $p < 0.01$ and *** $p < 0.001$; compared to the VEHICLE group.

Figure 2.

Glucocorticoid-induced DNA methylation changes in blood and hippocampus. Bisulfite-pyrosequencing was performed at three CpG dinucleotides in intron 1 (**a**) and four CpG dinucleotides in intron 5 (**b**) of the mouse *Fkbp5* gene in white blood cells of mice treated with corticosterone ("CORT"; N=12) and compared to those of vehicle-solution treated controls ("VEHICLE"; N=12). Bisulfite-pyrosequencing was also performed at three CpG dinucleotides in intron 1 (**c**) and four CpG dinucleotides in intron 5 (**d**) of the mouse *Fkbp5* gene in the hippocampus of mice treated with corticosterone and compared to those of vehicle-solution treated controls. Data are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: compared to the VEHICLE group.

Figure 3.

Correlation between mean plasma CORT vs. DNA methylation and gene expression. For all of the forty animals in the five groups (0, 25, 50, 75, or 100 $\mu\text{g/ml}$ of CORT), linear regression analysis was performed between the calculated mean plasma CORT levels versus: **(a)** DNA methylation of blood intron 1 CpG-2; **(b)** DNA methylation of hippocampal intron 5 CpG-4; **(c)** expression of hippocampal *Fkbp5*; and **(d)** expression of blood *Fkbp5*. R^2 , or square of the correlation coefficient and P-values are included for each comparison. For comparisons involving DNA methylation measurements, P-values have been adjusted by Bonferroni correction to reflect the number of CpGs tested.

Figure 4.

Correlation between DNA methylation of hippocampal intron 5 CpG-4 versus (a) DNA methylation of blood intron 1 CpG-2 and (b) expression of hippocampal *Fkbp5*. Relationship between DNA methylation of blood intron 1 CpG-2 versus expression in the hippocampus was also calculated (c). There was a relatively poor correlation between DNAm of intron 1 CpG-2 vs. expression in blood ($R^2 = 0.12$, $p = 0.026$, graph not shown). All of the forty animals, eight from each of the five treatment groups, are included. R^2 , or square of the correlation coefficient and P-values are included for each comparison. For comparisons involving DNA methylation measurements, P-values have been adjusted by Bonferroni correction to reflect the number of CpGs tested.

Table 1
Relationship between mean plasma CORT levels and DNAm of intronic CpGs

Correlation between mean plasma CORT levels and DNA methylation in blood and hippocampus. For both tissues, correlation values between DNA methylation versus four-week mean CORT levels for the two intronic regions are shown. R^2 , or square of the correlation coefficient and P-values are included for each comparison. P-values have been adjusted by Bonferroni correction to reflect the number of CpGs tested, and a “1” was assigned to those comparisons that were $p \geq 1$ following Bonferroni correction.

Tissues	<i>Fkbp5</i> Intron	CpG Position	Comparison to Mean Plasma CORT	
			R^2	P-value
Blood	Intron 1	CpG-1	0.52	2.2×10^{-6}
		CpG-2	0.68	7.1×10^{-10}
		CpG-3	0.17	0.12
	Intron 5	CpG-1	0.03	1
		CpG-2	0.30	0.003
		CpG-3	0.07	1
		CpG-4	0.08	1
Hippocampus	Intron 1	CpG-1	0.01	1
		CpG-2	0.04	1
		CpG-3	0.07	1
	Intron 5	CpG-1	0.24	0.020
		CpG-2	0.34	0.001
		CpG-3	0.30	0.003
		CpG-4	0.33	0.001

Table 2
Cross-tissue comparisons of DNAm between intron 1 CpGs in blood and intron 5 CpGs in brain

Inter-intronic correlation between blood and hippocampus DNA methylation. R² and P-values for intron 1 CpGs in blood vs. intron 5 CpGs in hippocampus are shown. P-values have been adjusted by Bonferroni correction to reflect the number of CpGs tested, which totaled 7 CpGs in blood × 7 CpGs in hippocampus, or 49 CpGs.

Tissues		Blood					
	<i>Fkbp5</i> Intronic CpGs	Intron 1					
		CpG-1		CpG-2		CpG-3	
Hippocampus	Intron 5	R ²	P-value	R ²	P-value	R ²	P-value
		CpG-1	0.17	0.42	0.26	0.23	0.087
		CpG-2	0.29	0.015	0.31	0.009	0.127
		CpG-3	0.23	0.077	0.37	0.002	0.034
		CpG-4	0.33	0.005	0.49	2.7×10 ⁻⁵	0.011