SPECIAL FEATURE

Glucocorticoid exposure during hippocampal neurogenesis primes future stress response by inducing changes in DNA methylation

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Prenatal stress exposure is associated with risk for psychiatric disorders later in life. This may be mediated in part via enhanced exposure to glucocorticoids (GCs), which are known to impact neurogenesis. We aimed to identify molecular mediators of these effects, focusing on long-lasting epigenetic changes. In a human hippocampal progenitor cell (HPC) line, we assessed the short- and long-term effects of GC exposure during neurogenesis on messenger RNA (mRNA) expression and DNA methylation (DNAm) profiles. GC exposure induced changes in DNAm at 27,812 CpG dinucleotides and in the expression of 3,857 transcripts (false discovery rate [FDR] ≤ 0.1 and absolute fold change [FC] expression ≥ 1.15). HPC expression and GC-affected DNAm profiles were enriched for changes observed during human fetal brain development. Differentially methylated sites (DMSs) with GC exposure clustered into 4 trajectories over HPC differentiation, with transient as well as long-lasting DNAm changes. Lasting DMSs mapped to distinct functional pathways and were selectively enriched for poised and bivalent enhancer marks. Lasting DMSs had little correlation with lasting expression changes but were associated with a significantly enhanced transcriptional response to a second acute GC challenge. A significant subset of lasting DMSs was also responsive to an acute GC challenge in peripheral blood. These tissue-overlapping DMSs were used to compute a polyepigenetic score that predicted exposure to conditions associated with altered prenatal GCs in newborn's cord blood DNA. Overall, our data suggest that early exposure to GCs can change the set point of future transcriptional responses to stress by inducing lasting DNAm changes. Such altered set points may relate to differential vulnerability to stress exposure later in life.

DNA methylation | glucocorticoids | hippocampal neurogenesis | gene expression | prenatal stress

Early life is one of the most important and sensitive periods during the development of an individual (1). Exposure to stress during this critical period, as early as prenatally, has been associated with a wide range of health problems later in life, such as increased reactivity to stress, cognitive deficits, and psychiatric and behavioral problems (1). In addition to alterations in fetal growth and neurobehavioral development (2), several studies have linked exposure to prenatal stress to structural and connectivity changes in the offspring brain (3, 4). One of the possible mechanisms mediating the negative effects of prenatal stress could be increased fetal exposure to glucocorticoids (GCs) (5–7). Over the course of normal gestation, there is a physiological rise of 2- to 4-fold in maternal GCs that is important for proper fetal growth and maturation. GC exposure of the fetus is tightly controlled by a number of mechanisms, including the metabolism of GCs in the placenta by the 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (8). Maternal prenatal stress, depression, and anxiety have been associated with biological changes that could increase fetal exposure to GCs above the required physiological levels. While a number of studies have reported increased plasma cortisol in women experiencing stress, depression, or anxiety during pregnancy, this effect is far from consistent (9). Maternal stress has been proposed to be associated with increased GC exposure of the fetus via reduced placental metabolism of cortisol to inactive metabolites by 11β-HSD2 (8). In addition, prenatal stress

Significance

Prenatal stress exposure is associated with a wide range of health problems later in life. This may be mediated in part via glucocorticoid (GC) exposure during fetal development known to impact neurogenesis and induce epigenetic changes. Using a human fetal hippocampal progenitor cell line to assess the effects of GCs, we observe that exposure to GCs early during neurogenesis results in lasting changes in DNA methylation (DNAm). Lasting DNAm alterations are associated with significantly enhanced transcriptional response to a subsequent GC exposure. Our data suggest that early exposure to GCs changes the set point of future transcriptional responses to stress by inducing lasting DNAm changes. Such altered set points may relate to differential vulnerability to stress exposure later in life.

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Data deposition: The data from the HPC gene expression microarray experiments and HPC methylation data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE119842, GSE119843, and GSE119846).

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has also been linked to changes in the offspring's hypothalamic–pituitary–adrenal (HPA) axis with increased and prolonged HPA axis reactivity consistently observed in animal studies, with similar effects, although less pronounced, described in humans (6, 9).

While likely not the sole mechanism explaining the adverse outcomes after exposure to prenatal stress, excessive exposure to GCs above the physiological level may contribute to the observed neurodevelopmental consequences. Although GCs are essential for fetal brain maturation, the developing brain has been shown to be especially vulnerable to excessive GCs, with lasting effects on cognition and cortical thickness reported (9). Effects of GC on neuronal progenitor cells have been identified as potential mediators of these effects (5, 10). Hippocampal neurogenesis, in particular, is of importance, as this brain region plays an essential role in regulating the negative feedback loop of the HPA axis. In mice, a single dose of dexamethasone (DEX), a synthetic GC, at embryonic day 15.5 decreased hippocampal volume and cell proliferation in the subgranular zone of the dentate gyrus in pups and impaired long-term depression and hippocampal neurogenesis in adult mice (11). In macaques, prenatal DEX exposure as well as prenatal stress reduced hippocampal volume and neurogenesis (12, 13). This is supported by in vitro data, where reduced neuronal proliferation and differentiation were observed in human multipotent hippocampal progenitor cells (HPCs) after DEX treatment as well as high doses of cortisol (14).

The molecular mechanisms of how prenatal GC exposure might induce these long-lasting changes on neurogenesis and brain structure are largely unknown. There is accumulating evidence, however, that epigenetic mechanisms are likely to play a major role in mediating these effects (15). At the molecular level, GCs bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors, which function as transcription factors and regulate gene expression in multiple tissues (16). In addition to altering gene transcription, GR activation can induce changes in DNA methylation (DNAm) (17, 18). Local demethylation at glucocorticoid-responsive elements (GREs) has been reported after GR stimulation, possibly mediated by activating base excision repair mechanisms (19). This reduction in DNAm likely changes accessibility of the DNA to transcriptional regulators and impacts future transcriptional responses (20).

Exposure to prenatal stress or GCs has been associated with persisting changes in DNAm in neuronal tissues and cells. In animal models of prenatal stress, lasting changes in DNAm in the hypothalamus or hippocampus have been reported in specific candidate genes (21, 22). Another set of studies has reported the impact of chronic administration of GCs on DNAm in adult mouse hippocampus as well as in a rodent primary neuronal cell line, both in candidate genes (17, 23) and at a genome-wide level (18, 24). Here, we extend these previous studies and systematically investigate the impact of GCs on genome-wide DNAm and gene expression in human HPCs undergoing neuronal differentiation (14). We examine how GC exposure at different stages, including proliferation, differentiation, and postdifferentiation, affects DNAm and whether these changes are persistent. A special focus is placed on developmental DNAm and gene expression trajectories and how these mechanisms are altered by GC exposure during different developmental periods as well as the interconnection of DNAm and gene expression changes across time. Finally, we map the observed epigenetic changes in HPCs to measures in developing human tissues and assess their potential as biomarkers for prenatal GC exposure.

Results

Effects of DEX Treatment During Neurogenesis. To assess the immediate and long-lasting effects of GR activation on gene expression and DNAm during neurogenesis, DEX treatment was applied at 4 different experimental time points in HPCs (Fig. 1*A* and *SI Appendix*, *SI Materials and Methods*) followed by messenger RNA (mRNA) and DNA hybridization onto Illumina arrays. Cells were first treated with DEX (1 μM) or vehicle (ethanol) only during the proliferation phase (Pro; 3 d) or during both the proliferation and neuronal differentiation phases (Pro-diff; 10 d). To assess long-lasting effects of DEX, cells treated during proliferation and differentiation stages were cultured for an additional 20 d without DEX (Pro-diff + washout [WO]). To compare DEX effects pre- and post-differentiation, cells were also treated with DEX or vehicle post-differentiation for 10 d followed by 20 d of WO (Post + WO). Clustering the HPCs gene expression profiles from the vehicle treatments with hippocampal gene expression data from embryonic to adult postmortem brains of the Human Brain Transcriptome atlas (25), we observed that these cells most resemble second trimester pregnancy hippocampal gene expression (*SI Appendix*, *SI Results* and Fig. S1).

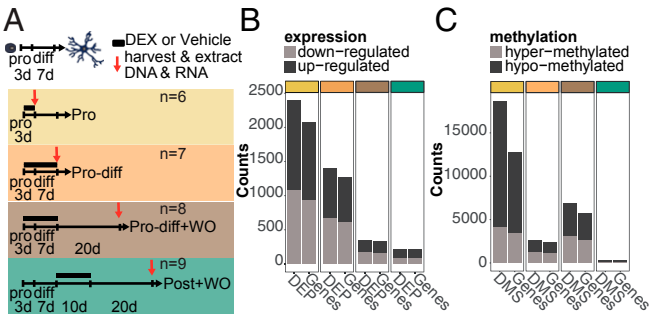

Fig. 1. DEX-induced changes in gene expression and DNAm across treatments. (*A*) Schema illustrating the different treatments with vehicle or DEX (1 μM) applied to HPCs across neurogenesis. Number of (*B*) DEPs and (*C*) DMSs induced by DEX across treatments. The bars on the left of each pair represent the number of significant probes from the array, and the bars on the right of each pair represent the number of genes mapped to these probes for each treatment.

Using immunohistochemistry, we previously reported that DEX treatment (1 μM) in HPCs decreases proliferation and differentiation of progenitor cells (14). Here, we predicted the proportion of neuronal, glial, and doublecortin (DCX)-positive cells across treatments using the CellCODE algorithm (26). As previously described (14), DEX significantly decreased neuronal and DCX-positive cell proportions during the proliferation and/or differentiation phases compared with the vehicle condition. However, the decrease in neuron, glial, and DCX-positive cell proportions did not persist after WO, indicating that these immediate effects are reversed within 20 d of additional culture (*SI Appendix*, *SI Results* and Fig. S2 *A* and *B*).

DEX-induced changes in gene expression and DNAm during neurogenesis. We identified significant gene expression changes in 3,512 unique transcripts (false discovery rate [FDR] ≤ 0.1 and absolute fold change [FC] ≥ 1.15) (Dataset S1) after DEX treatment across the 4 different time points. The majority of the changes were observed during proliferation (Pro; n = 2,389 transcripts or 68%) and differentiation (Pro-diff; n = 1,409 or 40%) (Fig. 1*B*). Only a small number of differentially expressed probes (DEPs) showed long-lasting DEX effects after WO both in pre- and post-differentiation treatments (Pro-diff + WO, n = 348 or 6% and Post + WO, n = 212 or 0.2%, respectively), indicating that, for the majority of the transcripts, changes were not maintained after the removal of DEX. Even though a much smaller number of DEPs was identified after WO (Pro-diff + WO), significant overlaps were observed with DEPs from the earlier time points (Pro vs. Pro-diff + WO, n = 80 and Fisher exact $P = 7.79 \times 10^{-5}$; Pro-diff vs. Pro-diff + WO, n = 70 and Fisher exact $P = 6.17 \times 10^{-11}$) but not with the postdifferentiation time point (*SI Appendix*, Fig. S3*A*). The same pattern was observed for analyses on the probe as well as at the gene level (Fig. 1*B* and *SI Appendix*, Fig. S3*B*).

Significant DEX-induced DNAm changes were identified in 27,812 unique CpG dinucleotides (FDR ≤ 0.1) (Dataset S1) across all time points. As for gene expression, the majority of differentially methylated sites (DMSs) were identified in cells treated in the proliferation stage (Pro; 65.5% of total DMSs), and minimal effects of DEX were seen when cells were treated postdifferentiation

(Post + WO; 1.1% of total DMSs) (Fig. 1*C*). In contrast to the effects on gene expression, a significantly larger proportion of CpG sites (24.4% of the total DMSs) showed long-lasting DNAm changes after WO ($P < 2.2 \times 10^{-16}$ based on the test for equality of proportions). This was not the case when the cells were treated after differentiation; here, a significantly lower proportion of DMSs (1.1%) was observed (Post + WO; proportion test $P < 2.2 \times 10^{-16}$). DMSs identified after WO (Pro-diff + WO) shared a significant overlap with DMSs identified at the earlier time points, especially when mapped to genes (at the gene level: Pro-diff vs. Pro-diff + WO, $n = 874$ and Fisher exact $P < 2.2 \times 10^{-16}$; Pro vs. Pro-diff + WO, $n = 3{,}194$ and Fisher exact $P < 2.2 \times 10^{-16}$) (*SI Appendix*, Fig. S3 *C* and *D*).

DEX-induced DMSs have distinct trajectories during neurogenesis. To follow up on our observation that changes in DNAm seem to be coordinated to some degree across developmental stages, we sought to determine whether these changes cluster in different DNAm trajectories across neurogenesis. We applied the Gene Activity in Patterns Sets (GAPS) algorithm (27) to identify the main trajectories by clustering the DNAm profiles of the top DMSs (FDR ≤ 0.1 and absolute DNAm change ≥5%, $n = 792$) (Dataset S1). We identified 4 trajectories across our experimental conditions, where 566 CpG sites were found to be uniquely associated with a specific trajectory (Fig. 2*A*). Across differentiation, DNAm levels at these DEX-responsive sites decrease (Fig. 2*A*, green trajectory) ($n = 71$ CpGs), increase (Fig. 2*A*, red trajectory) ($n = 127$ CpGs), or remain relatively stable (Fig. 2*A*, blue and beige trajectories) ($n = 330$ and 38 CpGs, respectively). The effects of DEX on DNAm, while significant for each single CpG for at least 1 time point, often showed differences in the direction (more or less methylated). In the blue and beige trajectories in Fig. 2*A* but not the 2 other trajectories, significant DEX-induced differences in average DNAm levels were observed (*SI Appendix*, Fig. S4). For 24% of the sites across all 4 trajectories, DEX-induced significant methylation changes that occur early in neurogenesis are maintained after the WO of DEX. At this time point (Pro-diff + WO), CpGs in the beige trajectory in Fig. 2*A* show the largest effects. We next mapped these 566 sites to their closest genes and performed enrichment analysis in Gene Ontology (GO) categories. Overall, genes mapped to these DNAm trajectories are involved in cellular and organ development, transcription, neurogenesis, and neuronal differentiation (Fig. 2*B*). For the majority of the genes (72%), we observe the expected inverse correlation between DNAm and mRNA expression profiles during proliferation and differentiation. For 142 of the transcripts mapped to the individual DNAm trajectories, DEX induced significant changes in mRNA expression during the proliferation and/or differentiation stages (Fig. 2*C* shows examples), but this was only observed for 18 transcripts after WO (Pro-diff + WO). The lack of concomitant mRNA expression and DNAm changes after the WO of DEX is also evident for all DMSs showing lasting DNAm changes (6,895 CpGs), where only 2.6% of the associated transcripts (4,368 transcripts) show long-lasting expression changes. The top DMS showing the largest long-lasting demethylation change (−20.1%, cg14284211) from the beige trajectory in Fig. 2*A* is located in the *FK506 binding protein 5* locus. Fine mapping of additional CpGs in this locus using targeted bisulfite sequencing shows similar long-lasting demethylation across multiple GREs of this locus (*SI Appendix*, *SI Results* and Fig. S5 and Dataset S1).

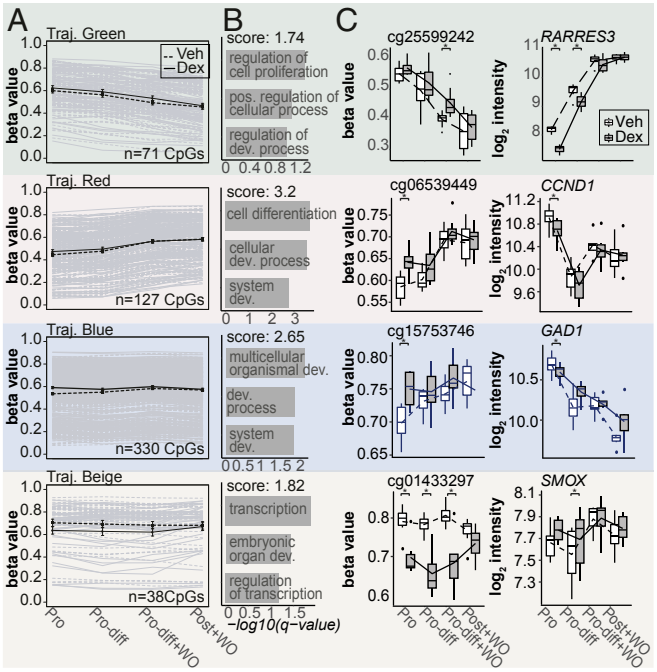

Fig. 2. Top DEX-induced DMSs cluster into 4 distinct trajectories during neurogenesis. (*A*) DNAm of the vehicle (dashed line) and DEX treatments across our experimental conditions for DMSs belonging to each of the 4 trajectories identified by the GAPS algorithm (*Top* to *Bottom* for all panels, the green, red, blue, and beige trajectories are depicted). The average DNAm and SEM overall sites within each trajectory appear in bold. (*B*) Top significantly enriched clusters of GO biological process terms for genes mapped to DMSs within each trajectory. (*C*) Boxplot of the methylation levels of a representative CpG site for each trajectory and its associated gene expression levels across treatments. *$P \leq 0.05$ from linear regressions comparing vehicle and DEX treatments. dev, development; pos, positive.

Functional annotation of DEX-induced DMSs. To annotate the biological functions of the DEX-induced changes observed, we performed multilevel ontology analysis combining the results from DNAm and mRNA expression to identify common GO biological processes between the 2 datasets. This analysis revealed an enrichment in pathways involved in neurogenesis as well as in the regulation of transcription across our time points (*SI Appendix*, *SI Results* and Fig. S7*A*). Interestingly, a set of pathways was exclusively associated with DNAm changes occurring in the Pro-diff + WO condition with associated gene expression changes at this time point as well as in the earlier time points. These were axon development, actin filament organization, negative regulation of cell proliferation, small guanosine triphosphate phosphohydrolases (GTPase) mediated signal transduction, and neuropeptide signaling pathways. This indicates that biological functions associated with lasting DNAm changes show earlier differential mRNA expression after DEX during proliferation and differentiation.

We next aimed to characterize the regulatory function of the genomic locations of our DEX-responsive DMSs. Using GR chromatin immunoprecipitation sequencing (ChIP-Seq) peaks from ENCODE lymphoblastoid cell lines exposed to DEX, we observed significant enrichment within GREs for DMSs in Pro-diff and Pro-diff + WO ($P_s < 0.001$, odds ratios [OR] = 1.59 and 1.25, respectively), while Pro and Post + WO treatments were not enriched for GR binding sites (Fig. 3*A*). Using the 15-states ChromHMM annotation of the Roadmap Epigenomics project for hippocampal tissue (28), we observed that DMSs of Pro, Pro-diff, and Pro-diff + WO treatments are enriched within enhancers and flanking active transcription start site (TSS) (Fig. 3*A*). Interestingly, an overrepresentation of multiple bivalent and/or poised states characterized by the presence of both activating and repressive histone marks was exclusively observed for the long-lasting DMSs (Fig. 3*A* and *SI Appendix*, Fig. S7*B*).

DNAm changes induced by prolonged GC exposure during neurogenesis are associated with enhanced responsivity of target transcripts to a subsequent acute GC challenge. The above presented analyses showed that early DEX exposure only leads to minimal lasting gene expression

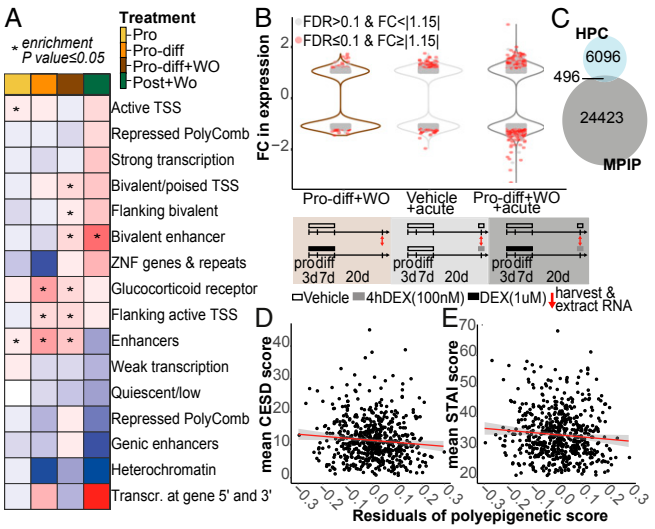

Fig. 3. Functionality of the long-lasting DMSs induced by GRE activation. (*A*) Heatmap of enrichment results for GR–ChIP-seq binding sites and predicted ChromHMM states for each treatment (colors display fold enrichment, and stars indicate significant permutation $P_s < 0.05$). (*B*) Violin plot showing the fold change (DEX – vehicles) in gene expression for each treatment condition of the 3,852 closest transcripts that map to CpGs showing long-lasting DNAm changes (Pro-diff + WO; n = 6,895 CpGs). (*Lower*) Schema illustrating the previous Pro-diff + WO treatment and the 2 acute challenge treatments applied. Significant transcripts for each treatment condition are marked in red. (*C*) Overlap of DEX-responsive DMSs in HPCs (Pro-diff + WO) and human peripheral blood cells of the MPIP cohort. Associations between maternal (*D*) depression ($\beta = -0.0015$, SE = 0.00066, $P = 0.022$) and (*E*) anxiety ($\beta = -0.0011$, SE = 0.00054, $P = 0.044$) during pregnancy and the polyepigenetic score computed for 817 newborns' cord blood DNA samples. ZNF, Zinc Finger; TSS, transcription start site; FC, fold change; FDR, false discovery rate; CESD, Center for Epidemiological Studies Depression Scale (CES-D); STAI, Spielberger's State-Trait Anxiety Inventory.

changes but to substantial changes in DNAm within regulatory regions. Such changes in DNAm may poise the target transcripts to a more exaggerated transcriptional response to a subsequent activation of the GR. To test this hypothesis, we used a combination of treatments with the early 10-d exposure to 1 μM DEX followed by the 20 d of WO and a single acute challenge of DEX at a lower concentration (100 nM) for 4 h and compared it with a single acute challenge of DEX in cells treated with vehicle during the early 10-d exposure (the schema in Fig. 3*B* and *SI Appendix, SI Results*). We focused this analysis on all transcripts that mapped to a CpG showing long-lasting DNAm changes (n = 3,852 transcripts nearby 6,895 Pro-diff + WO CpGs). We identified 702 transcripts (18.2%) with significant changes in gene expression after the additional acute challenge of DEX in comparison with cells treated with vehicle (Pro-diff + WO + acute; FDR ≤ 0.1 and absolute FC ≥ 1.15) (Dataset S1). This fraction was substantially higher than the one previously observed in Pro-diff + WO without the acute stimulation (n = 86 transcripts or 2.4%) or in cells exposed to the same acute challenge but treated with vehicle during proliferation and differentiation (n = 254 transcripts or 7.1%) (Dataset S1). In addition, these transcripts exhibited an overall larger magnitude of change in gene expression after the second acute challenge (mean absolute FC = 1.29 ± 0.19, range from −3.25 to 2.86) compared with minimal nonsignificant changes observed in the Pro-diff + WO treatment without acute challenge (mean absolute FC = 1.06 ± 0.07, range from −1.42 to 1.72, P value Wilcoxon test < 2.2×10^{-16}) or the acute challenge alone (mean absolute FC = 1.22 ± 0.1, range from −1.93 to 1.74, P value Wilcoxon test < 2.2×10^{-16}) (Fig. 3*B* and *SI Appendix*, Fig. S8). Together these results indicate that at least a subset of the long-lasting DMSs prime neighboring loci is more responsive to subsequent GR activation.

Interestingly, the lasting DMSs-associated genes with an increased response to subsequent DEX exposure (702 transcripts mapping to 1,282 CpGs) showed stronger enrichment among previously reported DMSs regulated during fetal development (29) compared with all of the long-lasting DMSs (Pro-diff + WO + acute permutation P = 0.004, OR = 1.3 compared with Pro-diff + WO permutation P < 0.001, OR = 1.23) (*SI Appendix, SI Results*).

Cross-Tissues Relevance of DEX-Induced Differential DNAm and Potential as Biomarker. Although GR-responsive changes in DNAm are likely to be largely tissue specific (20), overlapping DNAm changes have been reported in specific loci and may serve as biomarkers of exposure in peripheral tissues as observed in mice (23). To test this, we performed an enrichment analysis between the lasting DMSs in HPCs and DEX-associated DNAm changes in human blood cells from the Max Planck Institute of Psychiatry (MPIP) cohort (n = 113). In this dataset, we identified 26,264 CpGs with significant changes in DNAm (FDR ≤ 0.01 and absolute change in DNAm ≥ 2%) after correcting for confounders, including cell type proportions. We observed a significant overlap of 496 sites between these DEX-responsive CpGs (permutation P < 0.001 and OR = 1.1976) (Fig. 3*C* and *SI Appendix, SI Results*, Fig. S9, and Dataset S1).

We next wanted to test whether the lasting DNAm changes in HPCs with common DEX-induced changes in peripheral blood could serve as a biomarker for prenatal GC exposure in newborns. For this purpose, we used data from 817 newborns and their mothers within the Preeclampsia and Intrauterine Growth Restriction (PREDO) longitudinal cohort (30). We focused our analyses on pregnancy conditions related to higher prenatal GC levels: prenatal treatment with betamethasone, a synthetic GC, as well as the cumulative severity of maternal depression and anxiety symptoms throughout pregnancy. Using the overlapping 496 GC-responsive CpGs in blood and HPCs, we computed a weighted polyepigenetic score using an elastic net regression, which selected 24 CpG sites within 24 distinct loci. The weights were determined from the DEX-associated changes in peripheral blood (MPIP cohort), with the majority displaying reduced methylation after DEX (*SI Appendix, SI Results* and Fig. S9 and Dataset S1). Lower weights were associated with higher demethylation in blood after DEX exposure (β = 0.077, P = 0.04) (*SI Appendix*, Fig. S10). Applying this combined GC-responsive polyepigenetic score to DNAm measured in cord blood, we observed a significant association of this score with maternal anxiety ($\beta = -0.0011$, SE = 0.00054, P = 0.044) (Fig. 3*D*) and maternal depression ($\beta = -0.0015$, SE = 0.00066, P = 0.022) (Fig. 3*E*), with a lower polyepigenetic score observed in newborns exposed to higher depressive or anxiety symptoms. No significant association was seen with betamethasone treatment ($\beta = -0.0039$, SE = 0.019, P = 0.84), but here, only a small number of newborns (n = 35) were exposed to predelivery betamethasone treatment. However, as expected, the direction of the associations of betamethasone exposure, maternal depression, and anxiety with the score was the same.

Discussion

Using a human fetal HPC line, we observed that exposure to GCs during proliferation and differentiation, but not after the cells are differentiated, results in lasting changes in DNAm (Fig. 1*C*). These lasting DNAm changes are not correlated with strong baseline changes in gene transcription but with an enhanced responsivity of the target transcripts to a second GC challenge (Fig. 3*B*). This suggests that early exposure to GCs may have a lasting impact on nervous system development not only by altering proliferation and neuronal differentiation rates as previously reported (5) but also, by priming relevant transcripts to an altered transcriptional response on subsequent GR activation. The induction of such poised or metaplastic states could then contribute to the increased risk for behavioral problems and psychiatric disorders observed with prenatal GC exposure (9). In fact, the level of DNAm of these lasting DMSs is regulated

during human fetal brain development, especially for those linked to altered gene expression to a subsequent GC exposure. Moreover, when we used a subset of the DMSs showing lasting effects in HPCs and acute effects in blood to compute a GC-responsive polyepigenetic score in newborns' cord blood DNA, this score showed significant associations with maternal depression and anxiety (Fig. 3 *D* and *E*). This could suggest that the findings of our in vitro model may translate to human pregnancy and that DMSs with cross-tissue effects could serve as biomarkers for conditions associated with prenatal GC exposure.

Unique Functional Role of Lasting DNAm Changes. The lasting DMSs identified were distinct from the other DMSs, with only a limited overlap on the CpG level with DMSs after treatment during proliferation and differentiation (n = 180 overlapping CpGs or 2.6%) (*SI Appendix*, Fig. S3*C*). Indeed, unique GO terms relevant for the function of differentiated neurons were identified for genes mapped to these DMSs (*SI Appendix*, Fig. S7*A*). This suggests that, within the lasting DMSs, there could be at least 2 major categories, one related to differences in neurodevelopment and the other related to functional differences in mature cells. Prenatal GCs could thus not only impact neuronal proliferation and differentiation as such but also, change the sensitivity of more mature cells or tissues to stress exposure later in life. Indeed, an altered sensitivity to postnatal stressors after prenatal stress exposure termed metaplasticity has been proposed as a model for how prenatal environments may impact long-term risk trajectories (9, 31). This model suggests that different adaptive physiological responses to stress in individuals could be poised by prenatal stress (here we suggest via epigenetic mechanisms) but triggered by various postnatal environments, giving rise to the observed variety of short- and long-term phenotypic outcomes (*SI Appendix*, *SI Discussion* has a detailed description of this adaptive model).

In line with this model, the lasting DMSs were also enriched for a specific subset of chromatin marks (Fig. 3*A*), including bivalent/poised TSS and flanking bivalent and bivalent enhancers. Bivalent/poised chromatin states are characterized by the presence of both activating and repressive chromatin marks and are associated with paused RNA polymerase II that can be quickly released into productive transcription, a common feature of stress-responsive genes in yeast that is also observed in humans (32). Previous work investigating chromatin accessibility induced by GR activation identified a subset for which heightened sensitivity was retained as a "memory" of the hormone induction after withdrawal (33). In line with these observations, our results of enhanced gene expression changes after a subsequent GCs exposure for a subset of these long-lasting DMSs would suggest that these sites allow the cell to adjust its transcriptional response dependent on previous exposure. Although bivalency has been observed in differentiated tissues, it is important to note that the Roadmap data used for the enrichment analysis originate from bulk hippocampal tissue and that our DNAm profiles in HPCs are also from a cell mixture. Therefore, we cannot differentiate whether these sites are indeed localized at the bivalent/poised state of the same nucleosome or in different cells harboring one or the other chromatin marks. Nevertheless, the fact that the long-lasting DMSs are enriched among these regulatory marks and associate with altered expression after a subsequent exposure to GCs suggests a role for these sites in regulating or priming future gene expression responses to GCs, be it in a cell type-specific manner or within the context of a mixed tissue, with distinct GC sensitivities. These effects could thus alter the set point of ensembles of cells to future stress exposure.

Molecular Mechanisms Inducing DNAm Changes. What could be the mechanisms driving these lasting DNAm changes? GC-induced changes in DNAm may be direct downstream effects of GC action at the respective enhancer elements but may in part also be secondary to altered proliferation and differentiation observed after DEX treatment. In our previous work using a GR antagonist (14) as well as an inhibitor of *SGK1* (34), an activator of GR, we showed that both treatments block DEX-induced reduction in proliferation, providing evidence that at least some of these changes may be more directly downstream of GR activation. From our data, we observed that lasting DNAm was enriched in GREs, and for these sites only, we observed a larger fraction of demethylation vs. hypermethylation after DEX (*SI Appendix*, Fig. S7*B*). This is concordant with prior studies that have described local DNA demethylation at GREs with GR activation (18), likely mediated by induction of base excision repair mechanisms (19). However, DNA demethylation was not the rule for the lasting DNAm changes across all sites, with enhancers, bivalent/poised sites, and TSS flanking sites showing similar proportions of hypermethylation as well as demethylation (*SI Appendix*, Fig. S7*B*), similar to previous observations (18, 24). The fact that changes of DNAm were observed in both directions is also in line with our data showing that mRNA levels of both enzymes associated with demethylation (TET1) as well as remethylation (UHRF1) were affected by DEX after treatment during proliferation and differentiation (*SI Appendix*, Fig. S6). In contrast, changes in mRNA expression of TET1 and UHRF1 as well as differences in cell type proportions were not observed anymore after the 20 d of WO, although differences in DNAm are observed. These results suggest that long-lasting DNAm changes are not the result of strong and sustained global expression changes in epigenetic writers. However, they might result from a locus-specific recruitment and/or activation of these enzymes in response to GCs initiated in a small number of cells/alleles during proliferation and differentiation and continue to spread after the removal of DEX. Indeed, although not significant, we observed the same direction of changes in DNAm at the earlier time points (Pro and/or Pro-diff) for 54% of the long-lasting DMSs.

Cross-Tissues GC-Responsive CpGs as Biomarker for Prenatal Exposure. Lastly, we wanted to understand whether lasting changes in DNAm in our in vitro model would also be observed in human blood. While previous studies in mice have shown that GC-induced DNAm changes are mostly tissue specific (18), overlapping changes have been reported and may be aggregated in those GR-responsive enhancers with common functionality across tissues (23). We also identified a subset of lasting HPC DMSs that were also acutely responsive to DEX in peripheral blood (n = 496 CpGs) (Fig. 3*C*). In addition to being predictive of maternal stress exposures when combined into a GC-responsive polyepigenetic score, these cross-tissues CpGs were also significantly enriched in DNAm changes observed in cord blood of newborns exposed to predelivery administration of the synthetic GC, betamethasone, as well as maternal anxiety and depression (*SI Appendix*, *SI Results* and Dataset S1). A number of studies have reported that the 2 latter conditions might also be accompanied by increased fetal GC exposure by increasing maternal GC, decreasing placental GC metabolism, or activating the offspring's HPA axis (5, 6). Although not directly tested in our newborn cohort, maternal prenatal stress may also impact the newborn's DNAm profiles via other systems, such as immune activation with reciprocal interactions of the immune and stress systems (35, 36).

These DNAm changes in newborns may be markers for risk, as betamethasone exposure has been shown to be associated with mental health problems in children (7), and conditions associated with altered fetal GC exposure, including maternal depression and anxiety but also, infections, have been associated with a number of neurodevelopmental abnormalities (6, 9, 35). The fact that our cross-tissues GC-responsive polyepigenetic score significantly predicted both the severity of maternal prenatal depression as well as anxiety suggests that these sites could serve as biomarkers for prenatal GC exposure (Fig. 3 *D* and *E*). Lower scores reflecting more demethylation after GC exposure (*SI Appendix*, Fig. S10*B*) were associated with exposure to higher maternal depressive and anxiety symptoms over pregnancy. The direction of association together with the overlapping findings from prenatal betamethasone treatment would be in line with higher GC exposure in offspring of mothers with prenatal anxiety

and depression. Given that prenatal GC levels were not measured in the PREDO cohort, we cannot directly test this proposition. It is also important to note that, although we observed significant associations, the small effect sizes ($\beta = -0.0011$ for maternal anxiety and $\beta = -0.0015$ for maternal depression) are indicative that only a very small portion of the variance in symptoms is explained by the cross-tissues GC-responsive polyepigenetic score ($R^2 = 0.6\%$ for maternal anxiety and 0.3% for maternal depression) and would likely have small, clinically not relevant predictive power. Additional work is needed to further develop the score as well as replicate these associations in additional longitudinal cohorts with measure of GCs as well as early intervention studies to assess its ability to predict change in postnatal stress exposure.

Conclusions. Overall, our data suggest that GC-induced DNAm reflects a complex pattern of changes likely related to effects on proliferation and differentiation as well as lasting changes in more mature tissues. These lasting changes may specifically target pathways important for neuronal transmission and prime target genes to an altered responsivity to subsequent GC exposure. By this, prenatal exposure to GCs could not only alter neurodevelopmental trajectories but also, change the set point of stress reactivity of adult tissues. Together, these 2 factors could influence and increase the risk for psychiatric disorders.

Materials and Methods

Materials, experimental procedures, and data analysis for the culture and gene expression and methylation profiling of the immortalized, multipotent human fetal HPC line HPC03A/07 as well as methylation profiling in blood samples of the MPIP ($n = 113$) and PREDO ($n = 817$) cohorts are described in *SI Appendix, SI Materials and Methods*. The MPIP cohort study protocol was approved by the local ethics committee, and all individuals gave written informed consent. The PREDO study protocol was approved by the Ethical Committees of the Helsinki and Uusimaa Hospital District and by the participating hospitals. A written informed consent was obtained from all women. Data from the HPC gene expression microarray experiments (accession nos. GSE119842 and GSE119843) and HPC methylation data (accession no. GSE119846) were deposited in the Gene Expression Omnibus repository (37).

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