

# HYDROXYMETHYLATION AND DNA METHYLATION PROFILES IN THE PREFRONTAL CORTEX OF THE NON-HUMAN PRIMATE RHESUS MACAQUE AND THE IMPACT OF MATERNAL DEPRIVATION ON HYDROXYMETHYLATION

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**Abstract**—5-Hydroxymethylcytosine (5hmC) is abundant in the brain, suggesting an important role in epigenetic control of neuronal functions. In this paper, we show that 5hmC and 5-methylcytosine (5mC) levels are coordinately distributed in gene promoters of the rhesus macaque prefrontal cortex. Although promoter hydroxymethylation and methylation are overall negatively correlated with expression, a subset of highly expressed genes involved in specific cerebral functions is associated with high levels of 5mC and 5hmC. These relationships were also observed in the mouse cortex. Furthermore, we found that early-life maternal deprivation is associated, in the adult monkey cortex, with DNA hydroxymethylation changes of promoters of genes related to neurological functions and psychological disorders. These results reveal that early social adversity triggers variations in brain DNA hydroxymethylation that

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**Key words:** monkeys, hydroxymethylation, brain, early-adversity, rearing, maternal deprivation.

## INTRODUCTION

5-Hydroxymethylcytosine (5hmC), a modification of the 5-methylcytosine (5mC) base catalyzed by TET enzymes (Ito et al., 2010), is highly abundant in the brain (Kriaucionis and Heintz, 2009). The role of this modification is still unclear, but it has been postulated that it serves as an intermediate in DNA demethylation (Guo et al., 2011). However, the fact that 5-hydroxymethylation is persistently present in the brain argues that its role is distinct from that of DNA methylation. Here, we present and compare genome-wide mRNA, DNA hydroxymethylation and DNA methylation profiles of non-human primate rhesus macaque frontal cortices. We observed that the genome-wide promoter levels of 5hmC and 5mC are highly positively correlated and that their overall promoter levels are negatively correlated with expression. However, a subset of genes highly expressed is also highly methylated and hydroxymethylated. This suggests a different mode of regulation of expression by DNA methylation and hydroxymethylation of these genes.

A large body of studies in non-human primates has shown that various forms of social impoverishment have important long-lasting consequences on behavior and health; they have also demonstrated the effect of early experience independently of any genetic inheritance (Maestripieri, 1999, 2005). A long-standing challenge has been to identify the mechanisms that link these social exposures to persistent changes in physiology and behavior. We suggested that “epigenetic” mechanisms such as DNA methylation might be one component involved in embedding early social exposure, and particularly maternal care in the genome (Szyf, 2012) (Weaver et al., 2004; McGowan et al., 2009, 2011, Suderman et al., 2012). To confirm this hypothesis, we previously used a non-human primate model in which rhesus macaque monkeys are randomly assigned to different rearing conditions (Suomi et al.,

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Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ITPR2, 1,4,5-trisphosphate receptor, type 2; MeDIP, methylated DNA immunoprecipitation; MR, mother-reared; PFC, prefrontal cortex; SPR, surrogate peer-reared.

1976) since the mother–infant bond is one of the most fundamental early relationships in primates and is required for developing appropriate social skills (Cirulli et al., 2009). Peer-reared monkeys, as compared to normal mother-reared (MR) monkeys, exhibit abnormal behavioral traits that persist into adulthood (Ruppenthal et al., 1976). These pathological behaviors mirror what is seen in highly aggressive children and they are associated with differential risks for developing anxiety- and depressive-like disorders. This non-human primate model is therefore well suited to study the origins, developmental course, and long-term consequences of individual differences in physiological reactivity in a pathological state (Barr et al., 2003; Cirulli et al., 2009). We recently demonstrated that different rearing conditions in monkeys are associated with differential DNA methylation in both the prefrontal cortex (PFC) and T cells (Provencal et al., 2012). We therefore tested here whether a subset of these maternally deprived monkeys exhibited 5hmC differences from the maternally reared monkeys. We identified different hydroxymethylation levels associated with maternal and surrogate-peer rearing conditions at candidate gene promoters. Our results are consistent with 5hmC playing a role in epigenomic regulation.

## EXPERIMENTAL PROCEDURES

### Animals and rearing procedures

Samples were obtained from eight male rhesus monkeys (*Macaca mulatta*) at 7 years of age (born in 2000) that were housed born and reared at the LCE, NICHD breeding facility of the Animal Centre in Poolesville (MD, USA). The monkeys were randomly divided into two groups at birth resulting in different early-life social and rearing experience. The “MR” monkeys were raised by their biological mother in a social group, whereas “surrogate peer-reared” (SPR) monkeys were reared with an inanimate surrogate as well as daily socialization periods with age-mate peers. For the first month of life, the SPR monkeys were placed in a nursery until they were able to drink milk from a bottle by themselves at which point they were transferred to a cage with their surrogate mother. At approximately 7 months of age, animals were socially housed in large, mixed sex peer groups and were maintained under identical physical and social conditions during that extended period. Both rearing conditions are described in detail elsewhere (Shannon et al., 1998). All samples were processed and analyzed by experimenters blinded to rearing variables. All environmental conditions, procedures and handling of animals were in strict compliance with the Institutional Animal Care and Use Committee, and all experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Eight samples were used for DNA methylation analysis (4MR and 4SR) but only seven samples (3SR and 4MR) of DNA remained for the 5-hydroxymethylation analysis of candidate genes.

### PFC DNA and RNA preparation

Animals were sedated with ketamine and brought to a deep surgical plane of anesthesia with IV pentobarbital given to effect. A craniotomy was performed to expose the cerebral cortex and cerebellum, followed by a thoracotomy and perfusion through the left ventricle of the heart for 1.5 min with a chilled, oxygenated buffer solution. The rest of the brainstem was then exposed and the brain removed within 5 min. Prefrontal tissue was flash frozen in isopentane at  $-55^{\circ}\text{C}$  within 15 min of death and kept at  $-80^{\circ}\text{C}$  until DNA/RNA extraction. The tissue consisted mostly of dorsolateral and ventrolateral PFC rostral to the caudal end of the arcuate sulcus, dorsal to the cingulate sulcus and lateral to the lateral orbital sulcus. PFC DNA was extracted using the Qiagen DNeasy kit and RNA extraction using Trisol (Life Technologies, Burlington, ON, Canada), both following the manufacturer's protocol.

### Methylated DNA immunoprecipitation (MeDIP) and labeling

The MeDIP analysis was adapted from Keshet et al. (2006) and described in Provencal et al. (2012)). Briefly, 2  $\mu\text{g}$  PFC DNA of the eight monkeys was sonicated and methylated DNA was immunoprecipitated with 10  $\mu\text{g}$  of anti-5methylcytosine (EMD Millipore, Billerica, MA, USA). The input and bound fraction were then amplified in triplicate using the Whole Genome Amplification kit (Sigma-Aldrich, Oakville, ON, Canada). The amplified input and bound fractions were labeled for microarray hybridization with either Cy3-dUTP or Cy5-dUTP (Perkin Elmer, Woodbridge, ON, Canada) respectively using the CGH labeling kit (Life Technologies, Burlington, ON, Canada) following the manufacturer's instructions.

### Hydroxymethylated DNA enrichment and labeling

5hmC enrichment was performed using the Hydroxymethyl collector kit (ActiveMotif, Carlsbad, CA, USA) using DNA of six monkeys (four MR and two SPR) out of the identical eight monkeys used for the MeDIP (there was not sufficient DNA left from the other monkeys). Briefly, 1  $\mu\text{g}$  of DNA was fragmented (250–500 bases) using a bioruptor (Diagenode, Denville, NJ, USA) and was incubated in the presence of a  $\beta$ -glucosyltransferase enzyme and a modified UDP-glucose donor to create glucosyl-hydroxymethylcytosines. A biotin conjugate was then chemically attached to the modified glucose. Magnetic streptavidin beads were used to precipitate the biotinylated 5hmC DNA fragments. The input and bound fraction were then amplified using the Whole Genome Amplification kit (Sigma-Aldrich, Oakville, ON, Canada). The amplified input and bound fractions were labeled for microarray hybridization with either Cy3-dUTP or Cy5-dUTP respectively using the Agilent Enzymatic DNA Labeling Kit (Agilent Technologies, Mississauga, ON, Canada) following the manufacturer's instructions.

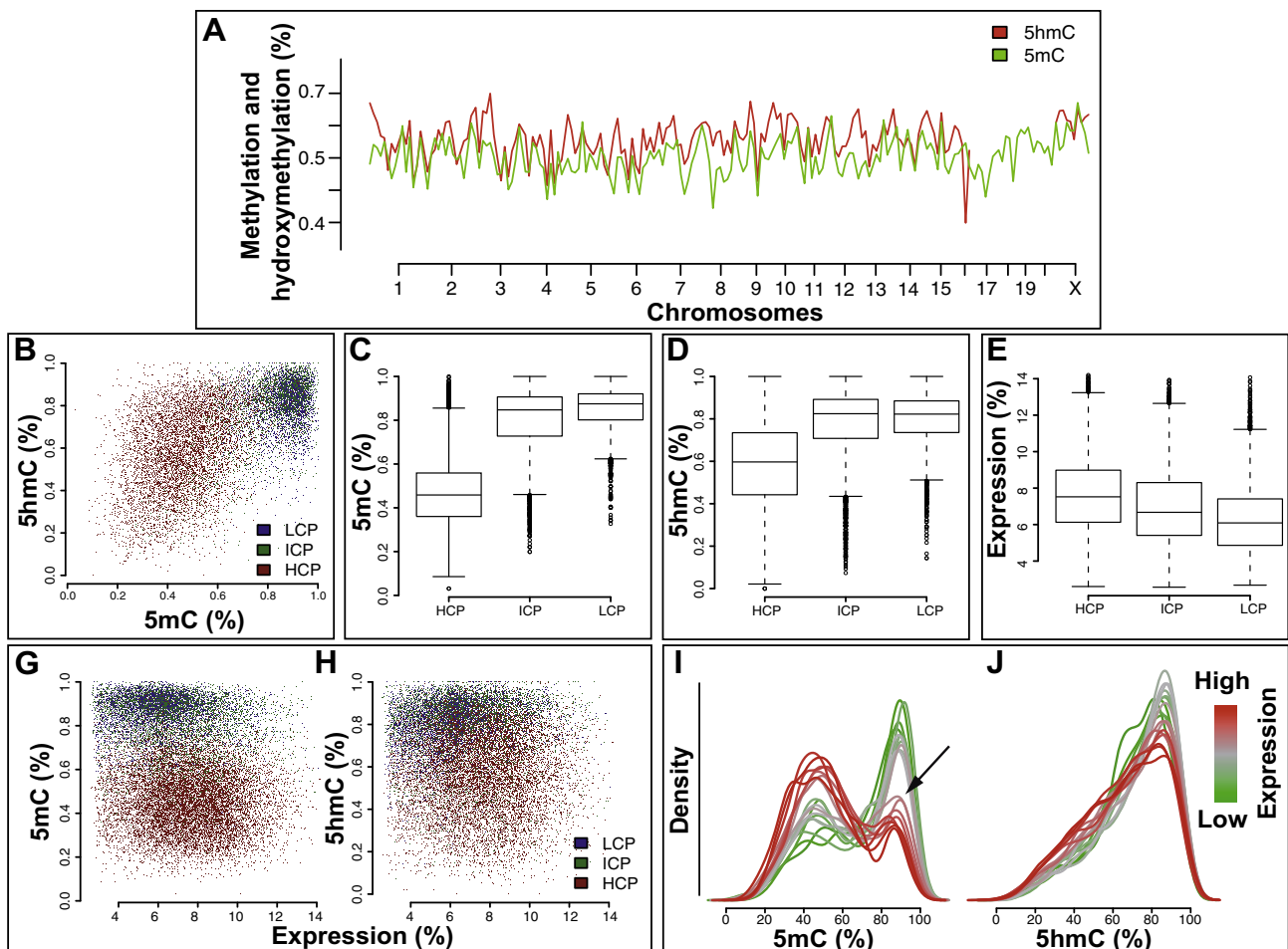
### MeDIP and 5hmC microarray design, hybridization, scanning and analysis

For MeDIP and 5hmC enrichment, custom 244 and 400 K promoter tiling array designs respectively were used for this study (Agilent Technologies, Mississauga, ON, Canada). Microarray probe sequences were selected to tile all gene promoter regions defined as the genomic interval from –2000 bp upstream to 400 bp downstream of each transcription start site as defined for the Rhesus Macaque by the Ensembl database (version 64.10) (<http://www.ensembl.org>).

All the steps of hybridization, washing, scanning and feature extraction were performed following the Agilent protocols for chip-on-chip analysis (5mC and for 5hmC enrichments). After microarray scanning, probe

intensities were extracted from scan images using Agilent's Feature Extraction 9.5.3 (MeDIP) and 10.5 (5hmC enrichment) Image Analysis Software. The extracted intensities were then analyzed using the R software environment for statistical computing (Team, 2007). Log-ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray and then microarrays were normalized to one another using quantile-normalization (Bolstad et al., 2003) under the assumption that all samples have identical overall methylation or hydroxymethylation levels.

The methylation or hydroxymethylation level of a probe or site, when estimated from microarray data, was obtained by applying a Bayesian deconvolution algorithm (Down et al., 2008). Fig. 1A illustrates these estimated levels across the genome.



**Fig. 1.** Correlation between DNA hydroxymethylation, DNA methylation and gene expression in the prefrontal cortex of rhesus macaques. (A) Overall promoter 5hmC ( $n = 6$ ) and 5mC ( $n = 8$ ) levels estimated from precipitation microarray data across the entire genome. 5hmC and 5mC are positively correlated. ( $R = 0.6$ ,  $p < 2E-16$ ) (Chromosomes 17–10 were not represented in the 5hmC arrays). (B) Correlation between hydroxymethylation and methylation. (C, D) Genomic distribution of 5mC and 5hmC across CpG islands. The levels of both 5mC and 5hmC show an inverse correlation with CpG density. The plots show the interquartile range. Below the box is the first quartile, above the box is the fourth quartiles, inside the box are the second and third quartiles. The whiskers stretch 1.5 times the interquartile range from the box. (E) Repartition of gene expression levels according to CpG density. Gene expression levels are positively correlated with CpG density. (G–J) Inverse correlations between promoter hydroxymethylation ( $R = -0.09$ ,  $p < 2E-16$ ) or methylation ( $R = -0.24$ ;  $p < 2E-16$ ) and gene expression levels estimated by microarray data. For figures I and J, genes are divided into 20 levels by expression percentiles (0–5, 5–10, ..., 95–100). Estimated distributions of promoter hydroxymethylation and methylation levels are shown for each expression percentile. Genes with low or no expression (represented in green) tend to have highly methylated promoters whereas genes with high expression (represented in red) tend to have lower promoter methylation. Gene exceptions (arrow) with high levels of methylation and highly expressed are indicated by an arrow. (LCP, low CpG density, ICP, intermediate CpG density, HCP, high CpG density). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 1F, H was obtained by partitioning the genes by expression percentiles (0–5, 5–10, ..., 95–100) derived from the expression microarrays. Each curve represents the distribution of estimated methylation or hydroxymethylation levels within the promoters of the corresponding gene partition.

The three-dimensional plots in Fig. 2 depict estimates of the variable corresponding to the vertical axis (e.g. expression level) based on values of two other variables. Estimates were made using a second-degree polynomial surface derived from the existing data using local fitting (LOESS).

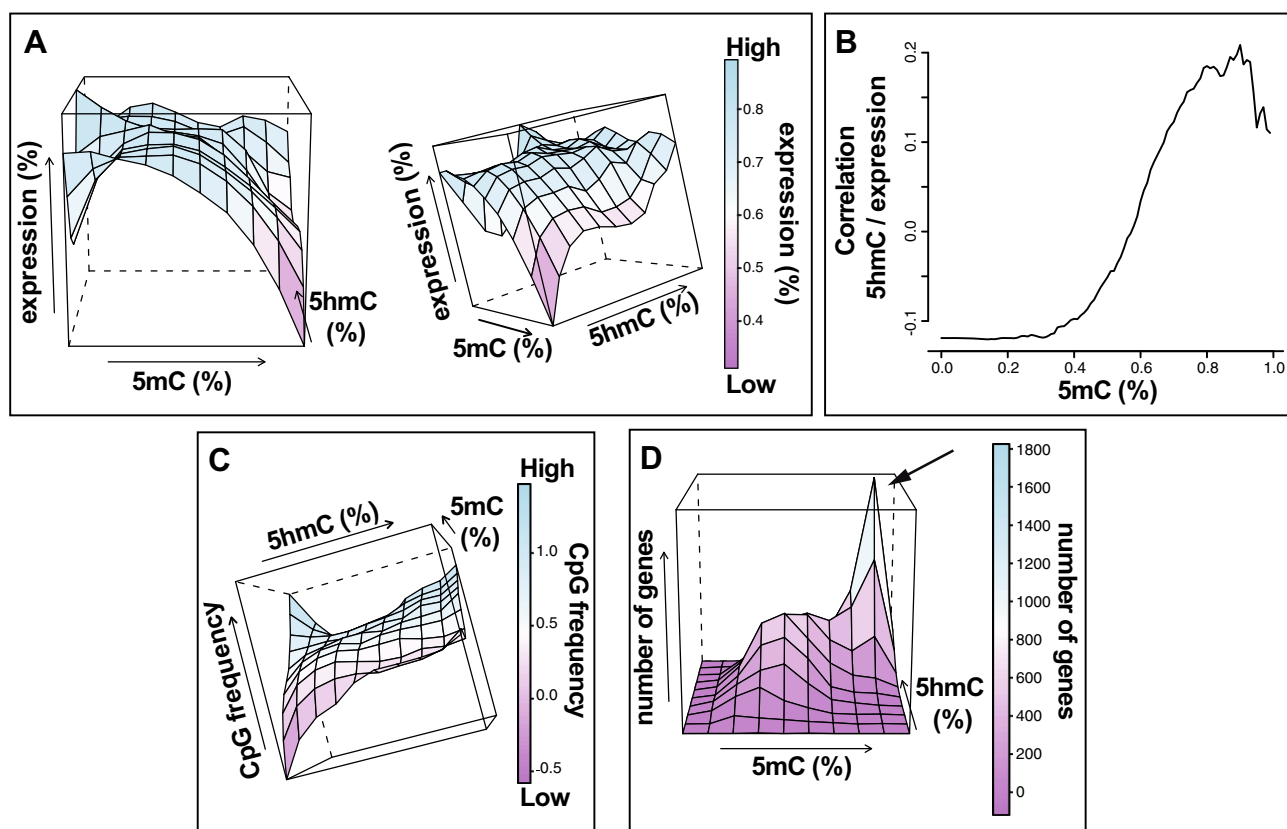
The normalized CpG frequency of a sequence is the frequency of CpG sites in the sequence divided by the expected frequency of CpG sites given the GC content of the sequence, i.e. the frequency of G nucleotides times the frequency of C nucleotides. The most common definition of CpG island requires that the DNA sequence has a normalized CpG frequency of at least 0.6.

For biological functions analyses, selected genes were overlaid on the global molecular network developed from information contained in the Ingenuity Pathway knowledge base ([www.ingenuity.com](http://www.ingenuity.com)). This network is composed of >3.5 million literature-based biological links between genes and bioactive molecules.

Sub-networks are built on genes of interest based on their connectivity within this global network taking into account the relative numbers of network eligible molecules, of molecules analyzed and the total number of molecules in Ingenuity's knowledge base. The top biological functions associated with each network are determined by querying the Ingenuity Pathways Knowledge Base for relationships between the genes in the network and the cellular and organismal functions they impact. The significance of the association between the network and those biological functions is scored using a *p*-value calculation. Functions having a *p*-value of less than 0.05 are displayed.

### cDNA microarray hybridization and analysis

mRNA expression quantification was already described in [Provencal et al. \(2012\)](#). Briefly, double-stranded cDNA was synthesized from total RNA from the PFC of all eight monkeys. *In vitro* transcription was performed to produce biotin-labeled cRNA using Affymetrix Gene Chip 3' IVT Express reagent kit according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). After fragmentation, 12.5 µg of cRNA was hybridized with GeneChip Rhesus Macaque Genome Array (Affymetrix) containing 47,000 genes. GeneChips were then scanned



**Fig. 2.** Integration of DNA hydroxymethylation, DNA methylation and gene expression in the prefrontal cortex of rhesus macaques. (A) The 3-D plot shows a general inverse correlation between methylation and expression. However, for highly methylated genes, a positive correlation between hydroxymethylation and expression is observed. (B) The positive correlation between hydroxymethylation and expression increases with methylation level. (C) Highly methylated genes display low and intermediate CpG frequency. Correlation between 5hmC and CpG frequency is mild for highly methylated genes ( $R = 0.02$ ,  $p = 0.1$ ). (D) Number of genes according to 5mC and 5hmC levels. An arrow indicates enrichment for a subset of highly hydroxymethylated and methylated genes.

with the GeneChip scanner 3000 (Affymetrix). Microarray probe intensities were normalized to each other using RMA (Irizarry et al., 2003). Expression differences between groups were then obtained by applying linear models implemented in the 'limma' package (Smyth, 2005) of Bioconductor (Gentleman et al., 2004) to obtain modified *t*-statistics and corresponding *p*-values. *P*-values were adjusted for multiple testing by converting them to false discovery rates.

### Hydroxymethylation differences

Quantitative real-time PCR of precipitated DNA samples (5hmC enrichment). Gene-specific real-time PCR validations of microarray were performed on the amplified and input bound fractions. Relative enrichment of triplicate reactions was determined after normalizing from the input fraction in each sample using the  $2^{-\Delta\Delta Ct}$  method. All data are expressed as group mean  $\pm$  SEM. Student's unpaired one-tailed *t*-test was used and the alpha level was set at 0.05. Graphpad 5 software (La Jolla, CA, USA) was used to perform statistical analysis.

### Mouse RNA-seq, hydroxymethylation and methylation data

Publicly available RNA-seq data obtained from the cortex of 8-week-old C57BL/6J mice was generated by the ENCODE project and downloaded from the Gene Expression Omnibus website (GEO accession: GSM1000563). Gene expression levels were estimated as the RPKM values obtained from the available GTF files. Hydroxymethylation and methylation levels of mouse frontal cortex were calculated from microarray data recently published (Massart et al., 2014). Only arrays of control mice ( $n = 9$ ) were used.

## RESULTS

### Genome wide analysis of 5hmC in the rhesus macaque frontal cortex

We created and analyzed genome-wide promoter hydroxymethylation profiles of PFC from six adult, male rhesus macaques. We compared these hydroxymethylation profiles with the recently published PFC methylation and mRNA expression profiles of adult, male rhesus macaques (Provencal et al., 2012). DNA hydroxymethylation and methylation profiles were obtained using a selective chemical labeling (Song et al., 2011; Szulwach et al., 2011b) and DNA immunoprecipitation enrichments respectively, followed by microarray hybridization.

### Integration of hydroxymethylation, methylation and mRNA expression profiles

Using a Bayesian deconvolution method to estimate hydroxymethylation and methylation levels from microarray enrichment data (Down et al., 2008), we found that promoter 5hmC and 5mC estimates across the genome are highly correlated ( $R = 0.6$ ,  $p < 2E-16$ ,

Fig. 1A, B). Both 5mC and 5hmC levels are inversely correlated with promoter CpG density (Fig. 1C, D) whereas mRNA expression levels are positively correlated (Fig. 1E). Surprisingly, although promoter 5mC levels are significantly negatively correlated with mRNA expression levels ( $R = -0.24$ ,  $p < 2E-16$ ; Fig. 1G–I), there was only a mildly negative correlation between mRNA expression levels and 5hmC levels ( $R = -0.09$ ,  $p < 2E-16$ ; Fig. 1H–J).

To better understand these relationships, we constructed a 3-dimensional plot of mRNA expression, methylation and hydroxymethylation levels. The plot indicates a positive correlation between 5hmC and mRNA expression for genes with highly methylated promoters (Fig. 2A). Indeed, as promoter 5mC levels increase, the correlation between mRNA expression and 5hmC levels also increases (Fig. 2B). For example, for genes with 5mC promoter levels above 0.75, the correlation is highly significant ( $R = 0.16$ ,  $p < 2E-16$ ). As shown in Fig. 2C, CpG frequency tends to be lower in the promoters of these genes and is only weakly correlated with 5hmC levels ( $R = 0.02$ ,  $p = 0.1$ ). The figure also shows that CpG frequency is, as expected, highest in genes with low 5mC levels. An analysis of the relationship between mRNA expression and 5mC levels (Fig. 1I) reveals that, for some genes, 5-mC and mRNA expression levels contradict their usually negatively correlated relationship. We found a strong positive correlation between 5-hmC and expression in these genes ( $R = 0.38$ ,  $p < 2E-16$ ). In particular, of the approximately 7000 genes with high 5mC levels (5mC > 75%), 1764 genes are highly expressed (expression greater than the median) (Fig. 1I, arrow points to this group of genes) and highly hydroxymethylated (Fig. 2D;  $p < 2E-16$ , Wilcoxon rank-sum test, median of 87% 5hmC compared to 74% overall). We use EG (Exception Genes) to denote this set of 1764 genes.

We calculated enrichments of biological processes in the EG genes to help define their physiological and molecular functions. The global analysis revealed enrichments for a number of neuronal functions (Table 1) and for neuronal or non-neuronal canonical pathways such as "Reelin Signaling in Neurons" ( $p = 9.55E-8$ , hypergeometric test) (Fig. 3), "CXCR4 signaling" ( $p = 3.02E-5$ , hypergeometric test), "FAK signaling" ( $p = 6.03E-5$ , hypergeometric test), "Axonal guidance signaling" ( $p = 4.37E-4$ , hypergeometric test), "CREB Signaling in Neurons" ( $p = 1.1E-3$ , hypergeometric test). We identified cell type-specific EG genes using a transcriptome database for astrocytes (152 genes), neurons (121 genes) and oligodendrocytes (124 genes) (Cahoy et al., 2008). Gene set analysis of the cell type-specific genes show enrichments in cellular pathways such as ERK/MAPK signaling ( $p = 1.3E-3$ ) for oligodendrocytes, RhoGDI signaling ( $p = 2.1E-5$ ) for neurons or clathrin-mediated endocytosis signaling ( $p = 1.4E-4$ ) for oligodendrocytes.

By establishing mouse cortex 5mC and 5hmC profiles using genome-wide data recently published (Massart et al., 2014) and comparing them with publicly available

**Table 1.** Biological functions enriched in genes with high hydroxymethylation, high methylation and high expression levels

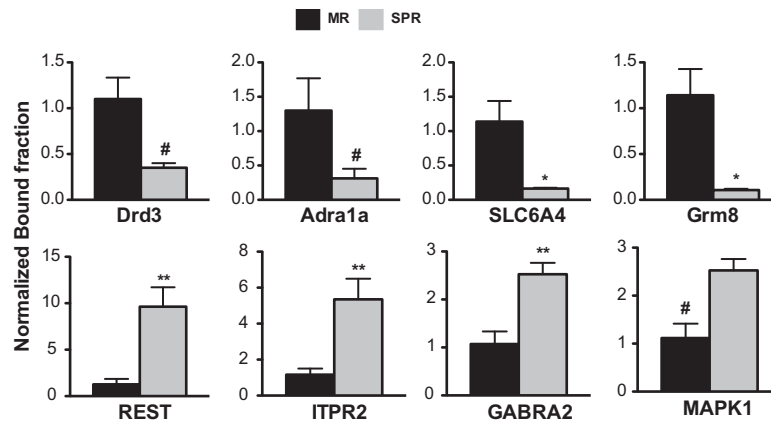
|                                                      |                                                | <i>p</i> -Values | Examples of subcategories           |
|------------------------------------------------------|------------------------------------------------|------------------|-------------------------------------|
| <i>Physiological system development and function</i> |                                                |                  |                                     |
| 1                                                    | Tissue morphology                              | 1.28E–05         | Morphology of nervous tissue        |
| 2                                                    | Immune cell trafficking                        | 3.86E–05         | Infiltration by macrophages         |
| 3                                                    | Cardiovascular system development and function | 8.03E–05         | Function of blood–brain barrier     |
| 4                                                    | Organismal development                         | 8.44E–05         | Development of cerebral cortex      |
| 5                                                    | Hematological system development and function  | 1.42E–04         | Infiltration by macrophages         |
| 6                                                    | Tissue development                             | 1.92E–04         | Neuritogenesis                      |
| 7                                                    | Hematopoiesis                                  | 2.09E–04         | Development of lymphocytes          |
| 8                                                    | Cell-mediated immune response                  | 5.46E–04         | T cells migration                   |
| 9                                                    | Embryonic development                          | 5.46E–04         | Development of cerebral cortex      |
| 10                                                   | Organ morphology                               | 1.10E–03         | Morphology of telencephalon         |
| 11                                                   | Behavior                                       | 2.71E–03         | Behavior                            |
| <i>Diseases and disorders</i>                        |                                                |                  |                                     |
| 1                                                    | Neurological disease                           | 3.63E–09         | Huntington's disease                |
| 2                                                    | Cancer                                         | 4.19E–06         | Neoplasia                           |
| 3                                                    | Immunological disease                          | 1.12E–05         | Autoimmune disease                  |
| 4                                                    | Inflammatory response                          | 1.42E–04         | Inflammatory response               |
| 5                                                    | Cardiovascular disease                         | 3.17E–04         | Hypertension                        |
| 6                                                    | Psychological disorders                        | 2.73E–03         | Late-onset Alzheimer disease        |
| 7                                                    | Developmental disorder                         | 4.81E–03         | Congenital anomaly of brain         |
| <i>Molecular and cellular functions</i>              |                                                |                  |                                     |
| 1                                                    | Cellular assembly and organization             | 4.88E–10         | Formation of neurites               |
| 2                                                    | Cellular movement                              | 2.89E–09         | Migration of brain cells            |
| 3                                                    | Cellular growth and proliferation              | 2.26E–07         | Proliferation of cells              |
| 4                                                    | Cell death                                     | 1.11E–06         | Apoptosis                           |
| 5                                                    | Cell morphology                                | 6.88E–06         | Outgrowth of neurites               |
| 6                                                    | Gene expression                                | 6.03E–05         | Transcription                       |
| 7                                                    | Cell signaling                                 | 8.58E–05         | Excitatory postsynaptic potential   |
| 8                                                    | Post-translational modification                | 8.58E–05         | Neurotransmission                   |
| 9                                                    | Protein synthesis                              | 8.58E–05         | Phosphorylation of proteins         |
| 10                                                   | Nucleic acid metabolism                        | 1.19E–04         | Assembly of protein–protein complex |
| 11                                                   | Small molecule biochemistry                    | 1.19E–04         | Metabolism of purine nucleotide     |
| 12                                                   | DNA replication, recombination, and repair     | 1.39E–04         | Uptake of neurotransmitter          |
| 13                                                   | Energy production                              | 1.39E–04         | Remodeling of chromatin             |
| 14                                                   | Cell-to-cell signaling and interaction         | 1.42E–04         | Infiltration by macrophages         |
| 15                                                   | Vitamin and mineral metabolism                 | 1.34E–03         | Homeostasis of Ca <sup>2+</sup>     |
| 16                                                   | Carbohydrate metabolism                        | 1.60E–03         | Metabolism of monosaccharide        |
| 17                                                   | Cellular compromise                            | 2.73E–03         | Depolymerization of filaments       |
| 18                                                   | Amino acid metabolism                          | 2.79E–03         | Phosphorylation of metabolism       |
| 19                                                   | Lipid metabolism                               | 4.24E–03         | Synthesis of sterol                 |
| 20                                                   | Molecular transport                            | 4.24E–03         | Uptake of neurotransmitter          |
| 21                                                   | Protein trafficking                            | 1.20E–02         | Localization of protein             |

RNASeq data generated by the ENCODE project (GEO accession: GSM1000563), we confirmed a negative correlation between the 5hmC levels ( $\pm 100$  bases from TSS) and mRNA expression ( $R = -0.11$ ,  $p < 0.0001$ , 68240 transcripts). However for genes with promoter methylation levels of at least 80%, 5hmC levels positively correlate with expression ( $R = 0.02$ ,  $p < 0.0097$ , 10288 transcripts). Moreover, the 651 genes highly expressed (greater than median), methylated ( $> 0.8$ ) and hydroxymethylated ( $> 0.75$ ) in the mouse cortex significantly overlap with monkey *EG* genes (79 genes,  $p = 5.08E-7$ , hypergeometric test). These mouse *EG* genes also present a low CpG frequency (0.28 on average). Gene set analysis of the mouse *EG* genes show, as for the monkey *EG* genes, the highest enrichments for neurological functions such as morphology of the nervous system ( $p = 5.31E-9$ ),

learning ( $p = 4.67E-8$ ) or morphology of the cells ( $p = 3.89E-8$ ). The mouse and monkey *EG* genes also share enriched canonical pathways such as “Reelin signaling” and potential regulators such as huntingtin.

#### Rearing-associated promoter hydroxymethylation differences

The number of monkeys in each rearing group analyzed in our 5hmC genome-wide study is small (four MR vs two SPR) and precludes us from establishing the genome-wide association of 5hmC with rearing conditions. The scarcity of DNA left from the other monkeys in the different rearing groups precluded increasing the number of monkeys that were analyzed by genome-wide arrays in the SPR group. However, we were able to determine the state of hydroxymethylation



**Fig. 3.** DNA hydroxymethylation differences in prefrontal cortex between mother-reared and surrogate peer-reared groups. QPCR analysis of DNA hydroxymethylation differences between rearing groups (SPR,  $n = 4$  – MR,  $n = 3$ ) in four promoters predicted to be less hydroxymethylated and four predicted to be more hydroxymethylated in the SPR animals by microarray analysis. Relative bound fraction concentrations obtained in triplicate by Q-PCR are shown for the eight genes (see methods). All error bars represent standard error of the mean (SEM). The symbol <sup>#</sup> denotes a  $p$ -value  $< 0.1$  and symbols <sup>\*</sup> and <sup>\*\*</sup> denote  $p$ -values  $< 0.05$  and  $< 0.01$  respectively from Student's  $t$  test.

of candidate genes that have already been associated with psychiatric disorders and/or perinatal adversity in three monkeys from the SPR group as well as four from the MR group using Hydroxymethyl collector kit followed by QPCR (Arnsten, 2000; Enoch, 2008; Le Foll et al., 2009; Castro et al., 2012; Rodenas-Ruano et al., 2012; Booij et al., 2013; Cao et al., 2013). We detected a decrease in hydroxymethylation in the SPR group compared to MR monkeys at promoters of the dopaminergic receptor 3, adrenoreceptor alpha 1, the serotonergic transporter, the glutamatergic receptor Grm8. In contrast, an increase in hydroxymethylation was measured at promoters of the inositol 1,4,5-trisphosphate receptor, type 2 (ITPR2), the GABAA receptor alpha 2, the transcriptional repressor REST and the MAP kinase 1 (Fig. 3). Interestingly, these promoters were not found differentially methylated between rearing conditions (Provencal et al., 2012).

## DISCUSSION

DNA methylation is involved in programming gene expression in differentiation and in response to environmental cues. Typically, methylation of the 5' regions of genes silences gene expression. DNA methylation could be further modified by an enzymatic reaction to generate the 5hmC marks. It has been proposed that 5hmC serves as an intermediate in a biochemical pathway leading to demethylation, either passively or actively (Ito et al., 2010; He et al., 2011; Inoue and Zhang, 2011; Maiti and Drohat, 2011; Zhang et al., 2012). Alternatively, it could serve as a stable epigenetic mark (Valinluck et al., 2004; Frauer et al., 2011; Yildirim et al., 2011; Hashimoto et al., 2012) that diversifies the DNA methylation signal. It is almost certain that 5hmC participates in gene expression programming since it is particularly enriched in enhancers, transcription factor binding sites and at promoters or gene bodies of actively transcribed genes (Jin et al., 2011; Stroud et al., 2011; Wu et al., 2011; Szulwach et al., 2011a). Additionally, 5hmC is highly abundant in

the brain, and in particular in the cortex, compared to other tissues (Kriaucionis and Heintz, 2009; Munzel et al., 2010; Li and Liu, 2011), suggesting an important role in epigenetic control of cerebral function. However, the role of 5hmC in genome regulation is unknown. We used a genome-wide approach to address the relationship between DNA methylation, DNA hydroxymethylation and gene expression in the cortex of a nonhuman primate. We also tested whether 5hmC is a responsive epigenetic signal by comparing promoter hydroxymethylation levels of candidate genes under differential rearing conditions.

Our study revealed that 5hmC and 5mC levels in promoters are correlated and are most abundant at regions of low and intermediate CpG content, similarly to ES cells (Booth et al., 2012; Yu et al., 2012). We also observed a positive correlation between gene expression and promoter 5hmC levels for genes with promoters of intermediate/low CpG content as already described in genes of low CpG content in human brains (Jin et al., 2011) and for gene bodies in the mouse frontal cortex (Lister et al., 2013), in contrast to the overall inverse correlation in ES cells (Xu et al., 2011). Most importantly, we describe for the first time a positive correlation between gene expression and promoter 5hmC levels for genes with high levels of promoter 5mC. This relationship was observed for both neuronal- and glial- specific genes and is therefore not restricted to a specific cell type. Interestingly, gene ontology analysis revealed that this subset of genes is involved in highly responsive signaling pathways and age-related neurodegenerative disorders such as Huntington's or Alzheimer's diseases, as previously reported for 5hmC (Song et al., 2011). This is of particular interest given that neurodegenerative pathways have been linked to oxidative stress and are probably highly reactive to the environment.

What is the role of the high methylation and high hydroxymethylation of the EG genes? The first possibility is that for this set of genes, the role of DNA methylation at promoters is different than its accepted role of

transcriptional silencer. Hydroxymethylation might play a similar or additional role in activating this subset of genes. The second possibility is that methylation silences *EG* genes in a fraction of cells and hydroxymethylation activates them in another fraction of cells. A third possibility is that hydroxymethylation counteracts the repressive action of adjacent methylated cytosines in the same alleles. However, there is no biochemical evidence to date, to our knowledge, that either DNA methylation or hydroxymethylation plays an active positive role in regulating promoter activity. On the contrary, recent data suggested that DNA hydroxymethylation silenced promoter activity in transient transfection reporter assays (Robertson et al., 2011). The fourth possibility is that the cortex is made of a heterologous cell population with a few cells where the *EG* genes are highly expressed, unmethylated and un-hydroxymethylated and a large population of cells where the *EG* genes are either methylated or hydroxymethylated but not expressed. This leads to measuring on average, with our genome-wide analysis, high levels of expression, methylation and hydroxymethylation for the *EG* genes. This is consistent with the relatively high proportion of cell type-specific genes in the subset of *EG* genes (22%) that are obviously expressed and repressed in different fractions of cells. It has previously been proposed that hydroxymethylation is found in promoter of genes that will later in development become demethylated (Lister et al., 2013). 5-Hydroxymethylcytosine might serve a similar purpose here; marking genes that will be demethylated in response to signals in the brain. It is therefore possible that the *EG* group consists of genes that are: – in a first subset of cells, methylated and not active – in a second subset of cells, hydroxymethylated ready to be transiently demethylated and activated in response to signals – in a third subset of cells, already activated and de(hydroxy)methylated. Further experiments are required to test these hypotheses. However, this is consistent with the gene set analysis showing that the *EG* genes are related to highly reactive signaling pathways.

Etiological studies of psychiatric disorders have traditionally focused primarily on genetic factors. However, it is likely that epigenetic factors are also highly important (Tsankova et al., 2007). It has been hypothesized that cues from the social and physical environments early in life can cause variations in epigenetic programming that serve as an “adaptive” response of the genome to the anticipated environment (Szyf, 2009). A misfit between the “adaptive” response and the real environment later in life would result in maladaptation contributing to the risk of developing psychiatric diseases (Meaney et al., 2007; McGowan and Kato, 2008). In mammalian development, the perinatal period might be a critical period when epigenetic programs are laid down resulting in changes in gene expression and to long-term influences on brain development and behavior. The role of DNA methylation variations in sustaining the effects of early environmental experience in mood-related disorders has been first demonstrated in the context of postnatal mother–infant

interactions (Weaver et al., 2004). Since then, other epigenetic modifications, such as modulations of promoters’ methylation states of the brain-derived neurotrophic factor (BDNF) or of the arginine vasopressin, were linked to the pathophysiology of mood disorders (Murgatroyd et al., 2009; Roth et al., 2009). Finally, associations of altered DNA methylation marks in the brain with mood related disorders in humans have been demonstrated (McGowan et al., 2008, 2009) and it has been established that differential rearing (maternal versus surrogate-peer rearing) of rhesus macaques results in genome-wide differential methylation in both PFC and T cells (Provencal et al., 2012). Unfortunately, the small number of monkeys from different rearing conditions used in our 5hmC genome-wide study prevents an analysis of the association of the genome-wide 5hmC profile, and more specifically of the *EG* genes, with rearing conditions. However, the differentially methylated genes associated with rearing do not overlap significantly with the *EG* genes (58 genes). This suggests that the *EG* genes might not be particularly affected by maternal deprivation. Therefore, we decided to measure promoter hydroxymethylation levels at candidate genes that have been associated with early-life adversity of psychiatric disorders but are not part of the *EG* genes (Arnsten, 2000; Enoch, 2008; Le Foll et al., 2009; Castro et al., 2012; Rodenas-Ruano et al., 2012; Booij et al., 2013; Cao et al., 2013). 5hmC differences at some of these genes in the maternally deprived animals (e.g., *DRD3*, *GABRA2*) might play a role in the cerebral cortex of adult monkeys and their propensity for addictive behavior or for their anxiety traits (Higley et al., 1991; Suomi, 1997; Enoch, 2008; Leggio et al., 2011). In contrast, *5HTT* is mainly expressed in the brain stem and the epigenetic modifications of its promoter in the cortex might not have obvious functional outcome. Nevertheless, the difference at the *5HTT* promoter in the cortex might be representing similar changes in other regions including the brain stem. The repressor element binding protein REST plays a major role during development. Interestingly, maternal deprivation impairs activation of REST and the developmental switch from GluN2B- to GluN2A-containing NMDA receptors (Rodenas-Ruano et al., 2012). Consistent with this hypothesis, elevated GluN1-, GluN2A- and GluN2B-containing NMDA receptors, resulting in an enhanced NMDA receptor function, were observed in adult offspring of mothers that had a low frequency of pup licking/grooming (Bagot et al., 2012). 5hmC changes at the promoter of *ITPR2*, an astrocyte-specific functional IP3R isoform, point to the potential involvement of glial cells in the behavioral disorders associated with maternal deprivation, such as depression (Suomi, 1997; Cao et al., 2013). This result also suggests that early adversity is associated with epigenetic differences in both neuronal-specific genes. These results suggest that hydroxymethylation might be considered as an additional epigenetic signal that mediates early-life events and their impact on the phenotype and possibly mental disorders. The possibility that 5hmC is driving epigenetic signals that mediate

mental health pathologies is obviously of high importance and needs to be further examined.

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