Neuroepigenetic programming in relation to the maternal environment: epigenome wide differential analysis of 5-methylation and 5hydroxymethylation in the brain PROGENIE UMR1198

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ABSTRACT

Epigenetic marks are established according to the environment and individual experiences. During brain development, they participate to the activity of neural circuits and contribute to the establishment and maintenance of several behaviours. Among them, cytosine methylation (5mC), one of the most studied modifications in the brain, is particularly sensitive to adverse environments. Recently, cytosine hydroxymethylation (5hmC), an abundant and stable mark derived from 5mC found in synaptic genes and enriched in fetal brain, has emerged as another major contributor to behavioral disturbances. These 2 important epigenetic markers constitute plausible molecular substrates in embedding the long-term effects of a maternal early experience on gene expression in brain structures linked to olfaction and odor-mediated behaviors that deserve to be studied. However, the quantification of 5hmC is still a bottleneck. This project aims to develop a method to quantify their variations in one important region of the brain, the olfactory bulb and to determine the respective contributions of 5mC and 5hmC in the modulation of cognitive functions related to olfaction observed in mice male offspring born to control dams or dams that have been fed a high-fat diet before a preconception weight loss (WL) (*Panchenko et al., 2019; Safi-Stibler et al., 2020*).

METHODS

Experimental Design and Sample Processing

Control (CTRL) or weight-loss mice having been fed a high-fat diet for 4 months before to be switched to a control diet 2 months before conception (WL) gave birth to offspring that were fed a standard control diet from weaning onwards. At 6 months of age, olfactory bulbs (OB) from adult male offspring born to CTRL (n = 4) or WL (n = 3) dams were collected for DNA extraction.

Library Preparation and Analysis

RRBS and oxRRBS libraries were prepared in parallel, following the protocol described in Perrier et al. (2018), with an additional DNA oxidation step applied specifically to the oxRRBS libraries (Figure 1). Sequencing data from RRBS and oxRRBS were processed using an integrated analysis pipeline incorporating custom in-house scripts (Python, R, Shell) and external tools (https://github.com/FAANG/faang-methylation/tree/master/RRBS-toolkit/). Standard methylation profiling was performed on the RRBS data to assess global levels of DNA methylation and hydroxymethylation, and on the oxRRBS data to quantify methylation specifically. To estimate 5-hydroxymethylcytosine (5hmC) levels, 5-methylcytosine (5mC) levels obtained from oxRRBS were subtracted from the total methylation levels obtained from RRBS at each CpG site across a common subset of 675,275 CpG positions. Differential methylation and hydroxymethylation analyses were then performed to compare WL and CTRL groups.

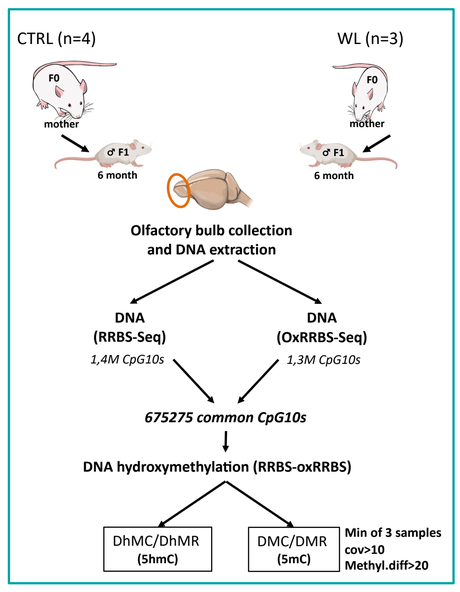


***FIGURE 1**: Overview of the experimental design and group comparison analysis within each dataset. The numbers of cytosines retained after data processing are shown.*

RESULTS

Characterization of methylation and hydroxymethylation data

	RRBS		OxRRBS	
	CTRL(n=3)	WL (n=4)	CTRL (n=3)	WL (n=4)
Number of read pairs (million)	67.4 ± 4.3	72.9 ± 5.5	70.4 ± 7.3	65.9 ± 4.4
Uniquely mapped reads (%)	67.2 ± 0.2	68 ± 1	68.3 ± 0.4	67.15 ± 1.4
Ambiguous reads (%)	21.6 ± 0.5	21.7 ± 0.9	21.9 ± 0.6	22.45 ± 1.3
Unmapped reads(%)	11.1 ± 0.3	10.3 ± 0.1	9.8 ± 0.2	10.4 ± 0.2
Bisulfite conversion rate (%)	99.97 ± 0.06	100 ± 0	99.97 ± 0.06	100 ± 0
Number of CpGs _10-500	1454454 ± 28861	1402671 ± 18203	1310124 ± 116507	1317646 ± 9174
Methylation, CpGs 10-500 (%)	38.9 ± 0.6	38.8 ± 1.15	34.9 ± 0.5	35 ± 1.6
hypomethylated (<20%) (%)	54.6 ± 0.8	54.6 ± 1.2	55.9 ± 0.9	56.3 ± 1.6
Intermediate (]20;80[%)	11.5 ± 0.404	11.5 ± 0.0957	18.3 ± 0.723	16.9 ± 0.829
hypermethylated (>=80%) (%)	34 ± 0.4	33.9 ± 1.1	25.9 ± 0.2	26.8 ± 2

***Table 1**: Characterization, mapping efficiency on the mice reference genome, coverage and average global methylation in RRBS (left) and oxRRBS (right) libraries*
Values are mean ± standard error of the means. CpGs10: CpGs covered by at least 10 uniquely mapped reads. Hypermethylated, intermediate and hypomethylated CpGs10 indicates CpGs10 with average methylation percentages > 80%, [20%; 80%], and < 20%, respectively.

Sequencing of RRBS and oxRRBS libraries yielded similar high-quality data.

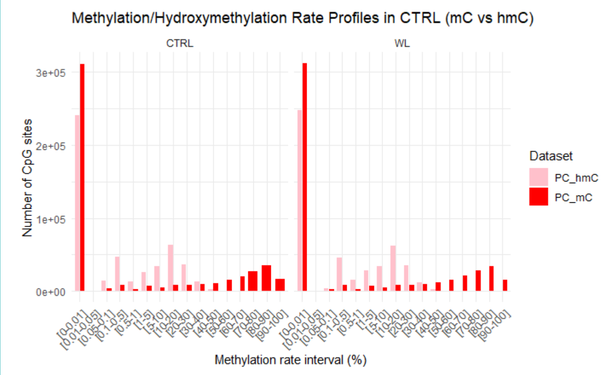
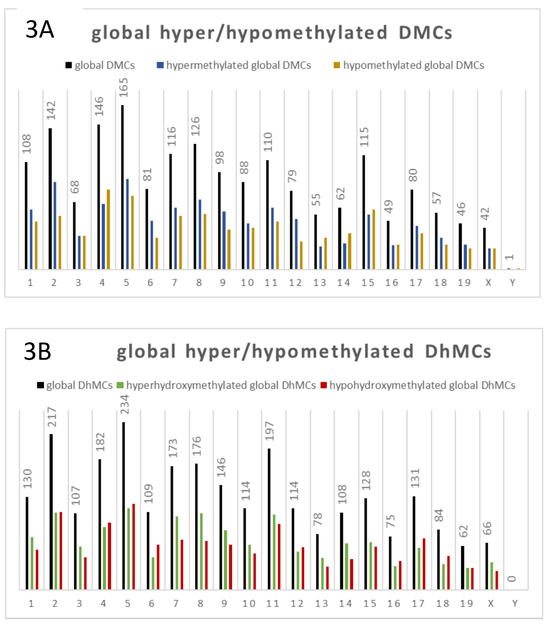


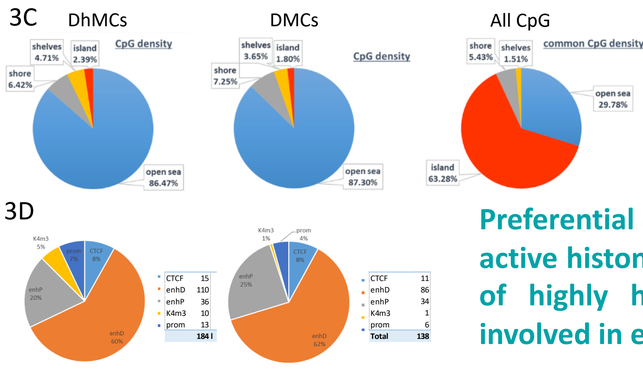
***Figure 2: Methylation/Hydroxymethylation Rate Profiles in CTRL and WL (mC vs hmC)**. CpG sites were grouped according to their methylation rates into predefined intervals.*

In both groups, average methylation level was 20.2% and average hydroxymethylation level was 5.5%. Only a few CpGs displayed high 5hmC levels (>60%) (Fig.2)
In CTRL, they map to genes linked to metabolism, synaptic modulation, and transcription. In WL, they are also located in genes related to plasticity, development, migration, and epigenetic regulation (not shown).

Differential analysis of methylation and hydroxymethylation in WL and CRTL samples



We identified **1,834 DMCs** located on 1301 genes, comprising 847 hypomethylated and 987 hypermethylated positions (**Fig. 3A**), and **2,631 DhMCs** located on 1418 genes, including 1,249 hypo-hydroxymethylated and 1,382 hyper-hydroxymethylated positions (**Fig. 3B**) in the WL group compared to the CTRL one. **DMCs and DhMCs were dispersed across the genome.**



DMCs and DhMCs are mostly located outside CpG islands, which is consistent with the epigenetic stability of islands between WL and CTRL (Fig.3C)

Preferential enrichment of DhMCs in promoters and active histone mark regions, echoing the association of highly hydroxyl-methylated CpGs with genes involved in epigenetic regulation (Fig. 3D).

Figure3: C, Pie charts of CpG, DMC or DhMC coverage in relation to gene features.
D, Pie chart showing the distribution of DMCs and DhMCs across regulatory genomic regions.

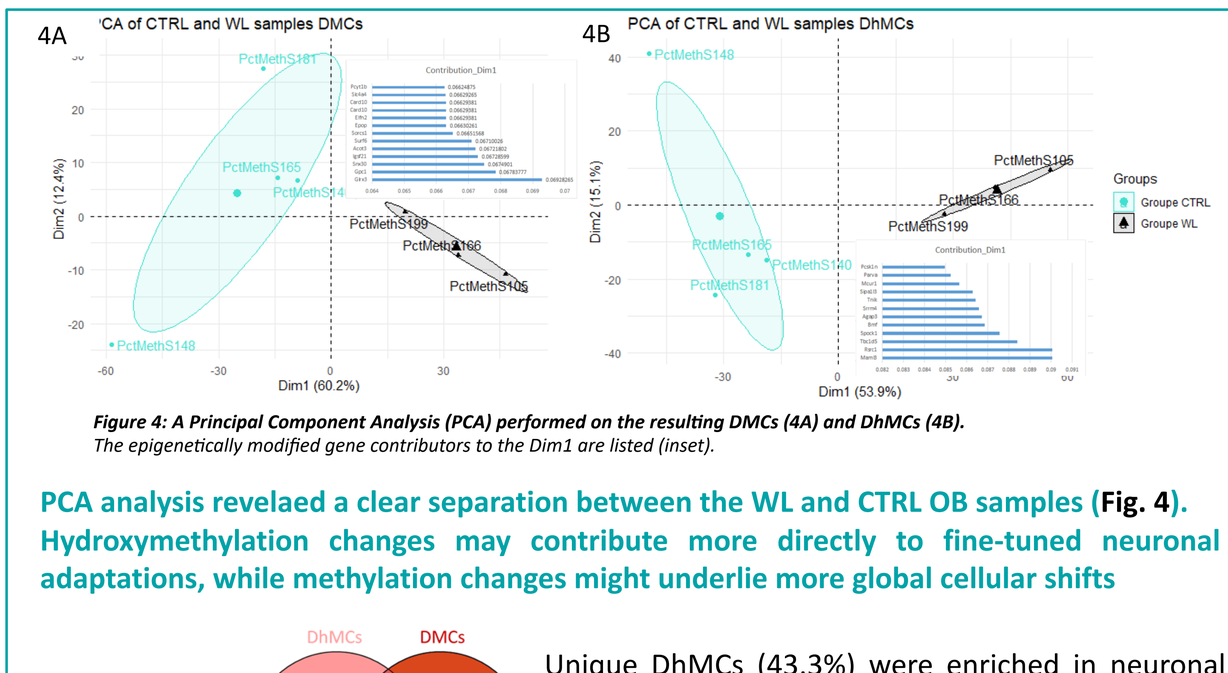
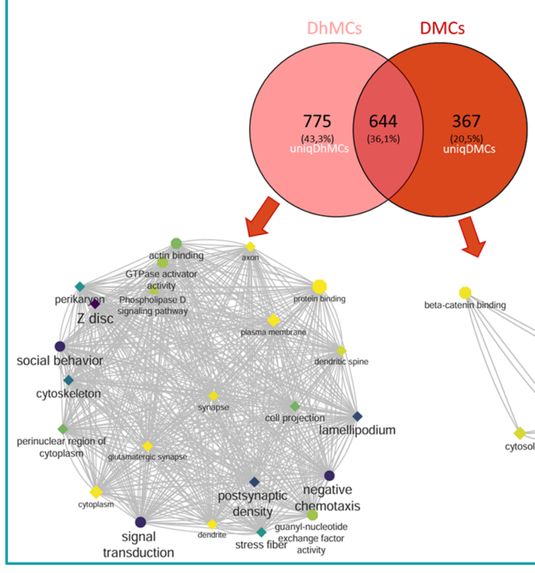


Figure 4: A Principal Component Analysis (PCA) performed on the resulting DMCs (4A) and DhMCs (4B).
The epigenetically modified gene contributors to the Dim1 are listed (inset).

PCA analysis revelaed a clear separation between the WL and CTRL OB samples (Fig. 4). Hydroxymethylation changes may contribute more directly to fine-tuned neuronal adaptations, while methylation changes might underlie more global cellular shifts



Unique DhMCs (43.3%) were enriched in neuronal genes, while unique DMCs (20.5%) were linked to transcription and protein binding regulation (Fig. 5).

The distinct profiles of unique positions confirm the need to analyze 5mC and 5hmC separately at each CpG site.

Figure 5: Venn diagram showing intersected overlap and differences between DMC and DhMC positions with the resulting network representation of enriched functional terms associated with uniqDMCs (right) or uniqDhMCs (left). Each node represents a GO term or pathway (shape = source). Node size reflects gene count; color and label size scale with $-\log_{10}$(FDR). Edge length indicates gene overlap (Jaccard index); edges connect terms sharing ≥1 gene.

DISCUSSION

We successfully developed an integrative framework to study brain epigenome plasticity, focusing on 5mC and 5hmC in the maternal environment. Our findings reveal that one-third of genes are co-regulated by both modifications. While 20.5% of DMCs are linked to transcription and protein binding, 43.3% of DhMCs are enriched in neuronal activity genes. High 5hmC levels in CpGs, especially in genes related to epigenetic regulation, and the preferential enrichment of DhMCs in promoters and active histone mark regions, suggest a critical role for 5hmC in brain gene regulation. These results underscore the need to distinguish the roles of 5mC and 5hmC to fully understand the influence of maternal environment on brain development.

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