Intra-individual methylomics detects the impact of early-life adversity

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Abstract

Genetic and environmental factors interact during sensitive periods early in life to influence mental health and disease via epigenetic processes such as DNA methylation. However, it is not known if DNA methylation changes outside the brain provide an 'epigenetic signature' of early-life experiences. Here, we employed a novel intra-individual approach by testing DNA methylation from buccal cells of individual rats before and immediately after exposure to one week of typical or adverse life experience. We find that whereas inter-individual changes in DNA methylation reflect the effect of age, DNA methylation changes within paired DNA samples from the same individual reflect the impact of diverse neonatal experiences. Genes coding for critical cellular–metabolic enzymes, ion channels and receptors were more methylated in pups exposed to the adverse environment, predictive of their repression. In contrast, the adverse experience was associated with less methylation on genes involved in pathways of death and inflammation as well as cell-fate related transcription factors, indicating their potential upregulation. Thus, intra-individual methylome signatures indicate large-scale transcription-driven alterations of cellular fate, growth and function.

Introduction

Experience, particularly during sensitive periods early in life, leaves indelible marks on an individual's ability to cope with life's challenges, influencing resilience or vulnerability to emotional disorders [1–5]. There is evidence that the mechanisms by which early-life experiences influence the function of neurons and neuronal networks involve modifying the repertoire and levels of gene expression via epigenetic processes [1–4,6–11]. Among epigenetic processes, changes in DNA methylation of individual genes and at the genomic scale have been reported, and these generally correlate with gene expression [2,6,12–14]. However, it is not known if DNA methylation changes might provide a useful "epigenetic signature" of early-life experiences in an individual child. Such a readily-accessible measure might serve as a biomarker for vulnerability or resilience to mental illness. Obviously, it is not possible to repeatedly sample DNA from brain cells in humans in order to assess DNA methylation changes for predicting and preventing disease. Therefore, current approaches employ peripheral cells including white blood cells (WBC) or buccal swabs (mixed epithelial/WBC), which are available repeatedly and noninvasively. Here we tested the feasibility of using peripheral DNA samples to assess the impact of diverse neonatal experiences on an individual by directly comparing two samples collected at different time points from the same individual rat in groups exposed to distinct early-life experiences with defined onset and duration. We have previously established that these diverse experiences provoke specific phenotypic outcomes later in life [4,15,16]. Specifically, we imposed "simulated poverty" by raising pups for a week (from postnatal day P2 to P10) in cages with limited bedding and nesting materials (LBN). This manipulation disrupts the care provided by the rat dam to her pups and results in profound yet transient stress in the pups, devoid of major weight-loss or physical changes. This transient experience provokes significant and life-long deficits in memory and generates increases in emotional measures of anhedonia and depression [15–17].

Here we tested if adversity during a defined sensitive developmental period in rats leads to a detectable epigenomic signature in DNA from buccal-swab cells. We obtained intra-individual epigenomic signatures of early-life adversity using reduced representation bisulfite sequencing (RRBS) [18] to identify changes in DNA methylation profiles. Comparisons were made both between two samples from an individual rat (P2 vs P10) and between samples from rats subjected to the two neonatal experiences. We found that assessing the overall methylation profile of samples

enabled detection of age and development effects [17,19], distinguishing P2 samples from those obtained on P10, but did not separate the two groups of pups based on their experience. In contrast, the changes in DNA methylation in two samples obtained from the same rat enabled clear differentiation of the control vs the adverse experience, likely by obviating large inter-individual variance. Thus, our findings establish the feasibility of identifying markers of adverse experiences that portend risk or resilience to mental illness, with major potential translational impact.

Results

Methylation level changes across individuals reflect postnatal age rather than early-life experiences

We obtained a mix of epithelial & white blood cell DNA from individual rat pups twice, on P2 and on P10, using buccal swabs (Methods). We obtained buccal swabs rather than peripheral white blood cells for three reasons. First, the swab, lasting seconds, is much less stressful than a painful needle prick to obtain peripheral blood, and this stress might influence methylation in itself. Second, this approach provides a more direct comparison with human studies where ethical reasons preclude needle pain whereas buccal swabs are routinely implemented [20,21]. Finally, several studies found that DNA methylation profiles in buccal swab cells are more similar to patterns from several brain regions than methylation profiles in white blood cells [21–24]. Following the initial samples collected on P2 from a group of naïve pups, the rats were divided into two groups: one was exposed to simulated poverty. The other was reared in a typical environment for one week. Samples from individuals in both groups were collected again on P10. We examined for intra-individual epigenomic signatures of early-life adversity and compared both P10 samples from groups with two divergent neonatal experiences as well as the changes in methylation levels between matched samples from the same individual rat (P2 vs P10; Figure 1A).

DNA methylation status was assessed using RRBS, with libraries sequenced to an average of 20 Million mapped reads, and we reliably detected an average of 482,000 CpGs in both samples of the same individual (Figure S1; Methods). We performed differential methylation analysis between P2 and P10 for each individual and identified 3417 significantly differential methylation regions (DMRs) after coalescing CpGs within 100 basepairs that were shared in at least two individuals from each experience group (Figure 1B). We analyzed the DNA methylation levels of these DMRs in P2 and in P10 for both the control and adversity-experiencing (LBN) groups across

individuals using k-means clustering and observed substantial changes in DNA methylation level during the one-week interval in both control and LBN (Figure 2A). The DNA methylation levels of individual samples clearly distinguished rats at different ages (Figure 2A). We further performed principal component analysis (PCA) on the percentage of DNA methylation of these DMRs and found that individual samples were separated by age using the first three principal components (up to 62.1% variances explained), indicating a substantial change in DNA methylation associated with age (Figure 2B, S2, S3). The separation by age still held when cohort effects were considered (Figure S4-6). These data demonstrate that development and age modify the buccal swab methylome [19,23,25,26]. PC2, which accounts for 20.7% of the variance, was the dominant component distinguishing samples of different ages (Figure 2B). We found that the PC2 DMRs with most positive weights for predicting the increased age (P10) had reduced methylation level in P10 while DMRs with most negative PC2 weights had increased methylation level in P10 (Figure S2, S3). However, the PCA analyses of the P2 and P10 methylome profiles did not separate the control group from the adversity-experiencing group (Figure 2C). Thus, whereas the level of DNA methylation in buccal swabs reflect an epigenetic signature of age, it provides little information about antecedent life experiences.

Intra-individual changes in methylation can distinguish early-life experience

To probe the impact of the early-life adversity experienced by an individual on DNA methylation patterns of the same individual, we explored intra-individual fold changes in methylation (referred to as "delta methylation", defined as $\log_2$(P10/P2) of the methylation level of P2 and P10 from the same individual) rather than the absolute value of methylation levels for each pup by taking advantage of the two samples collected immediately before and after a week of imposed adversity. We clustered and aligned these delta methylation profiles in both early-life experiences. We then examined the intra-individual methylation changes in detail and found that the patterns of changes in methylation within an individual were distinct depending on group assignment (Figure S7). PCA on delta methylation changes of individual samples reveals that delta methylation within an individual distinguished the control and LBN groups (Figure 3A, S8). Specifically, the fourth principal component (PC4), accounting for 4.2% of the variances, distinguished most LBNs from controls (Figure 3A, S8A). To examine the basis of the separation between LBNs and controls by PC4, we examined the relative contribution of individual DMRs to

the overall difference in PC4, and, guided by the slope of the weight distribution, selected a cutoff threshold at +/- $2.5x10^{-2}$ to identify 193 DMRs with the most positive weights and 225 DMRs with the most negative weights (Figure 3B, C). Importantly, the adverse and control experiences differentially changed levels of methylation in an experience-specific manner. Thus, within the top-predicting DMRs, the prediction of belonging to the LBN group (afforded by the intra-individual methylation changes in 193 most positive weights associated DMRs) involved relatively more methylation, compared with controls (Figure 3B). In contrast, intra-individual changes in the 225 most negative weights associated DMRs suggested generally less methylation level in LBN than the ones in control experience (Figure 3C). These results indicate that intra-individual changes in methylation-level profiles before and after a defined experience provide a novel epigenetic signature that identifies the nature of the experience.

Downstream significance of differential methylation resulting from age and experience

The paragraphs above demonstrate that profiles of absolute levels of DNA methylation in mixed epithelial / white blood cell samples from buccal swab can separate pups by age, whereas the nature of methylation changes in the same individual (delta methylation) distinguishes different early-life experiences. Whereas the relations of levels of methylation and of gene expression are not linear, we sought to examine the genes involved in methylation changes related to age as well as those related to experience.

Principal component (PC2) distinguished rats by age (Figure 2B). We focused only on analyzing the control group to obviate potential effect of the adversity experience (Figure S3). We found that 249 DMRs contributed the majority of the overall differences in PC2, which predicted the age being P10 in controls. Of these, 135 DMRs were less methylated while 114 DMRs were more methylated in P10 (Figure 4A, Table S1). We performed gene association analysis on these top-predicting DMRs of P10 and found that our 135 most positive weight PC2 DMRs were associated with 105 genes while the 114 most negative weight PC2 DMRs were associated with 91 genes. These age-related genes could be clustered into distinct functional categories (Figure 4B). In general, genes with decreased methylation level, predictive of augmented gene expression, were involved in energy metabolism (Man1c1, B4galt4, Mcart1, Mrc2, Ampd3, Arhgef17), cytoskeleton and trafficking (Fry, Krt42, RGD130731, Itga6, Fbxo9), receptors and ion channels

(Htr2a, Scarf2, Kcnip1, Traf3) and cellular responses to growth hormones (Fgfr3, Ltbp1, Net1). Gene ontology analysis identified gene clusters involved in response to injury, regulation of growth and ion transport (Figure S3C). By contrast, genes with increased methylation (i.e., expected to be less expressed with increasing age) were enriched in transcription (Otx1, Pax9, Dlx4, Irx4, Satb2, Nr2f2) and kinases (Srcin1, Map3k6, Atp8b4, Jak3) (Figure 4B). These findings suggest that age-related methylation changes are strongly involved in developmental processes in the neonatal organism.

In order to characterize the genes influenced by the adverse LBN experience, we performed gene association analysis on the top-predicting PC4 DMRs of LBN (Figure 3) and found that the 193 most positive weight PC4 DMRs were associated with 135 genes while the 225 most negative weight PC4 DMRs were associated with 165 genes (Figure 4C, Table S2). The 193 most positive weight DMRs had generally more methylation compared with controls, which suggests reduced gene expression (relative repression) after the LBN experience. The corresponding 135 genes coded for critical cellular enzymes and interacting proteins essential for normal metabolism and growth such as cellular cytoskeleton and trafficking (Sys1, Map3k8, Plekhg5) and cellular metabolism (Mrpl23, Hs6st1). Other genes within this group include Sipa1, Eif3k, Ttll5, Mark2, Ralgapa2, Net1, H6pd, Tet3, Dapp1, Sulf2, Ppp1r21, Dusp7, Nudt9. In addition, these PC4 positive weight associated genes coded for receptors/ion channels and transmembrane signaling proteins (Chrna9, Grik5, Gpr39, Nrp2, Fzd5, Pcnx1, Cd83), response to inflammation (Pdcd6ip, Tnfrsf1b, Card10, Traf3, Cxcr4) and transcription factors responding to growth factors (Sim2, Meis1, Lrrfip, Rai1) (Figure 4D). The combined expected repression of these genes would lead to disruption of typical growth, metabolism and maturation processes that are fundamental to the developing organism. In contrast, the 165 genes with the most negative PC4 weight DMRs were generally less methylated in the adversity experiencing rats (i.e., predicted to be expressed at relatively higher levels) and were strongly enriched in homeobox genes involved in very early cell specification (Six2, Hoxb5, Satb2, Six1, Dlx1, Nkx2-3), as well as other transcription factors and corepressors (Tfap2c, Skor1, Tbx3, Gata3, Tbx4, Hr). In addition, the group included genes involved in apoptosis and inflammation, including Dapk1, Gdnf, Mog and Tnfaip2 (Figure 4D). Increased expression of these genes would indicate a reversion to earlier, more primitive cell state and evidence of inflammation and reprogramming, perhaps to avoid death.

Discussion

We find here that comparing cohort-wide DNA samples obtained at different developmental ages reveals the signature of age and development on the peripheral methylome, as widely reported. However, these inter-individual analyses do not distinguish the divergent impacts of diverse experiences that take place during the intervening developmental epoch. By contrast, paired samples from the same individual before and after an adverse or typical developmental experience enable clear distinction of each of these experiences: we identify epigenetic 'scars' and 'kisses' that, at least in the rodent, precede and predict later-life emotional functions.

Although it is known that early life experiences drive gene expression changes and thus further influence the maturation of brain and other organs in mammalian individuals, our knowledge about specific epigenetic regulations involved into these processes are limited. Among epigenetic regulations, DNA methylation is known to correlate with gene expression changes. However, it is not known if DNA methylation changes might provide a useful "epigenetic signature" of early-life experiences in an individual child. Therefore, this study addresses two critical questions to understand the nature of DNA methylation changes in early life experiences: (1) does a short period of early postnatal life change methylation patterns in individuals? (2) can methylation changes be used to distinguish individuals who had experienced early-life adversity? Consistent with previous studies, we find that simple comparison of methylation levels across a cohort cannot distinguish rats with different early life experiences, though the signature of growth/ age is apparent. We further develop a novel approach and demonstrate for the first time that intra-individual changes in methylation patterns can robustly distinguish individuals with adverse experiences from those reared in typical conditions, thus potentially serving as a predictive signature in individuals.

While cognizant of the complex relation of DNA methylation levels and gene expression, we speculate here on the downstream consequences of the potential expression changes of gene families and individual genes that differentiate adverse and typical development. Among the genes differentially methylated in the groups of rats studied here, many overlapped in the LBN and control groups, suggesting that they are modified primarily by age rather than experience. Importantly, our PCA analyses of intra-individual methylation changes identified the PC4 genes

that were differentially methylated in the P10 LBN rats compared to the same rats on P2, but that were not affected in P10 vs P2 controls. These genes might then provide information about the processes associated with the early-life adversity experience that might carry long-term consequences.

Indeed, analyses of the top contributing genes to the distinction of having survived adversity in P10 rats was revealing: in LBN rats, there was a striking enrichment of increased-methylation (indicative of reduced expression) in genes carrying out typical processes of metabolism, trafficking and growth. In contrast, there was an expected overexpression (reduced methylation) of gene families associated with inflammation, death and reversion to more primitive developmental states. These seem to be orchestrated by differentially methylated transcription factors. *How* the adversity experience provokes these changes is unclear, and may involve molecular signals, including hormones and nutrients that modulate the complex enzymatic processes that govern DNA methylation status [27–29].

In summary, we show here the influence of a short epoch of adversity during a developmental sensitive period on intra-individual rodent methylome. In future studies, it would be exciting if this DNA methylation signature of early life adversity applied in human neonates and infants.

Figures

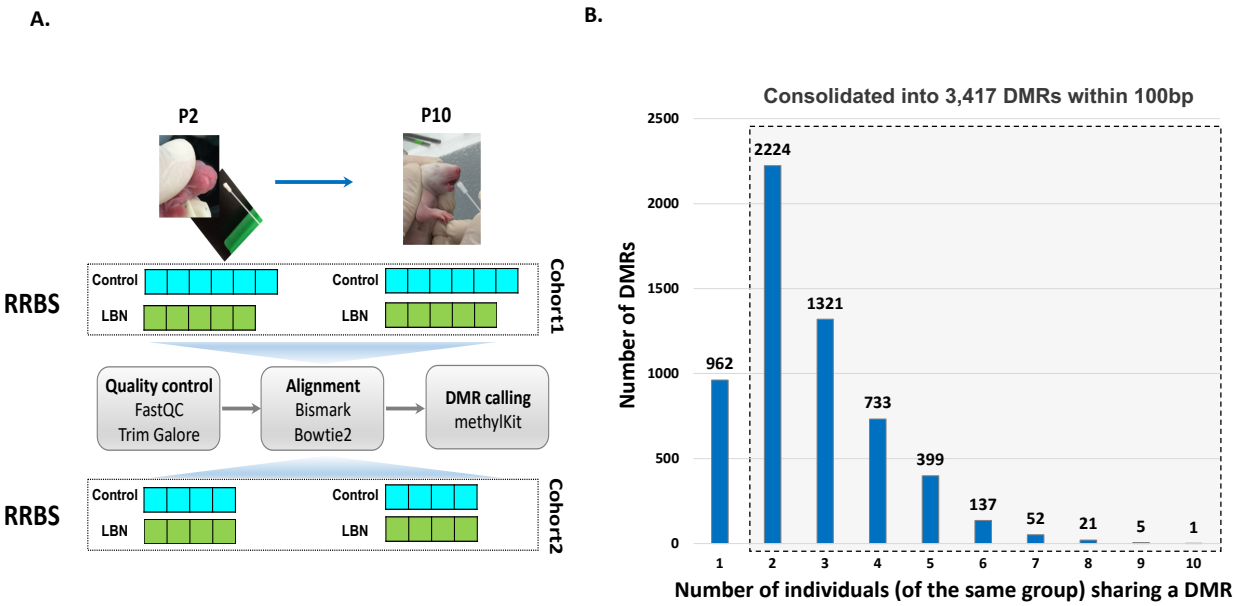

Figure 1. Experimental design and DMRs calling across individuals.

(A) Experimental design and analysis pipeline. **(B)** Histogram of the number of significant differentially methylated regions (DMRs) based on the number of individuals sharing the same experience. RRBS = reduced representation bisulfite sequencing; LBN- limited nesting and bedding cages, a paradigm of simulated poverty and early-life adversity. P2, P10 = postnatal days 2,10.

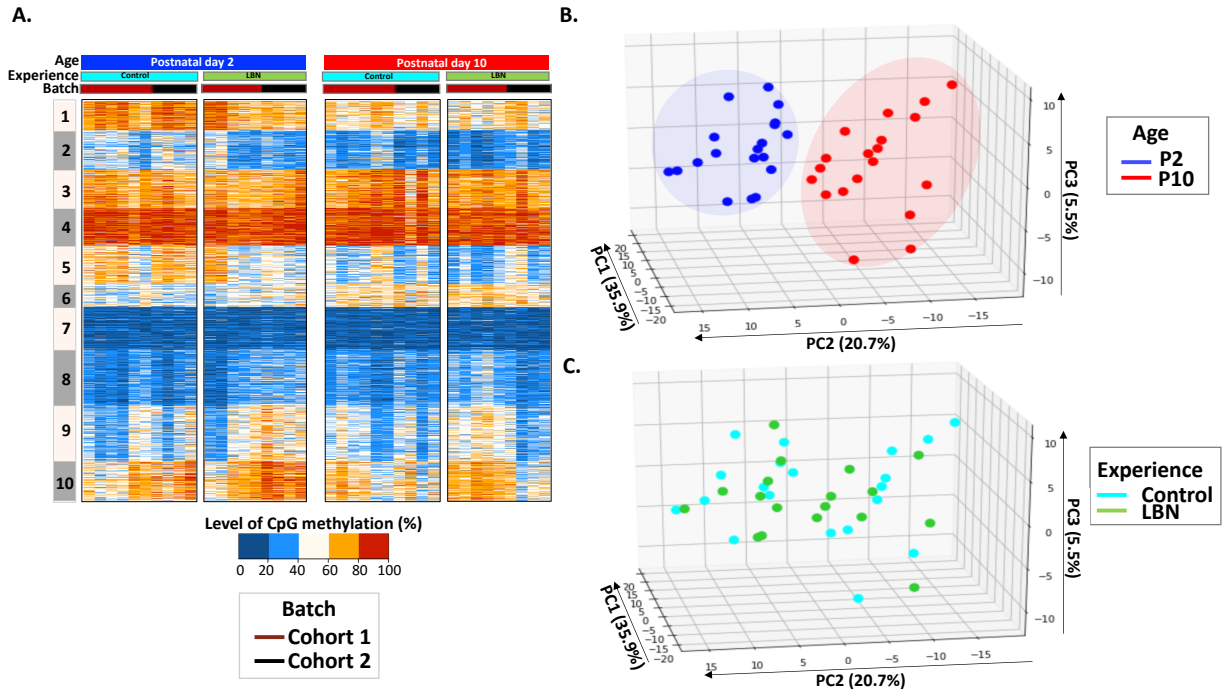

Figure 2. Separation of individuals by age by profiles of methylation levels on significantly differentially methylated regions (DMRs).

(A) Heatmap of CpG methylation percentage on the 3,417 DMRs identified in Figure 1, and showing individual samples. The profile is presented as 10 clusters that are identified using K-means clustering. Blue, low methylation percentage; orange, high methylation percentage. **(B)** Principal component analysis (PCA) of methylation profiles of individual samples, focusing on the 3,417 significant DMRs. Individual samples are labeled by age, P2, blue; P10, red. **(C)** Principal component analysis (PCA) of individual samples focusing on the same DMRs. Individual samples are labeled by experience, Control, cyan; LBN, green.

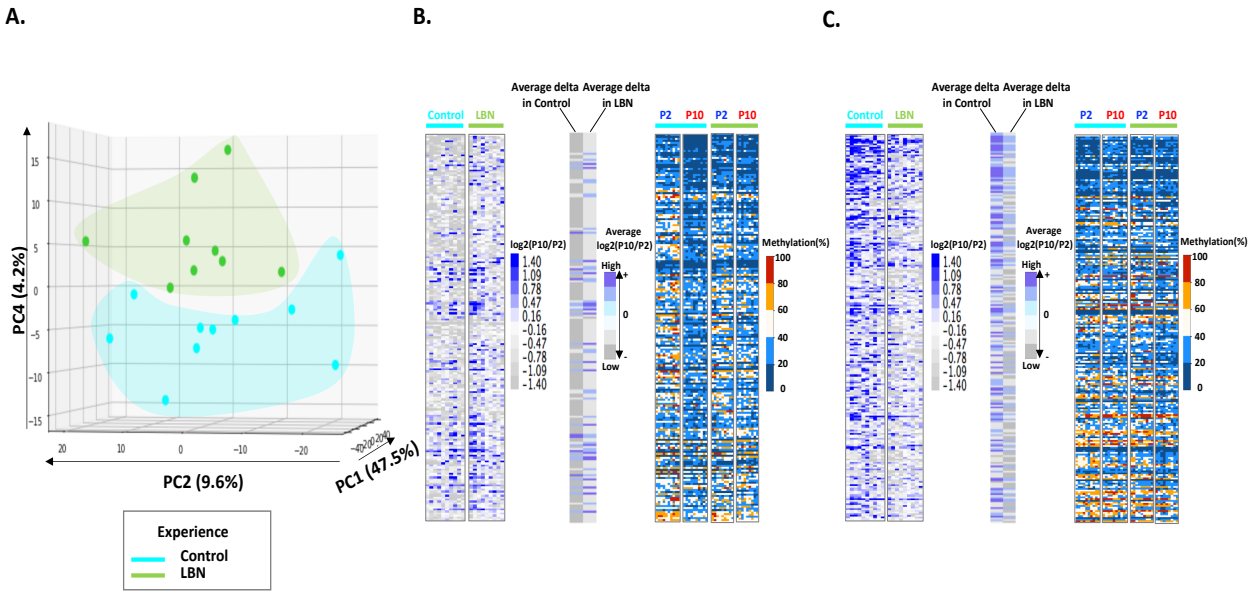

Figure 3. Intra-individual methylation analysis of significant DMRs separates individual rats by experience

(A) Principal component analysis (PCA) performed on the difference in methylation levels (delta methylation) between P10 and P2 (log2(P10/P2)) of an individual pup. We focus on the on 3,417 identified DMRs, and label individuals by experience, Control, cyan; LBN, green. PC4 provides the discrimination. **(B)** Delta methylation profile, average delta methylation and absolute methylation levels (in percent) of 193 DMRs with most positive weights in PC4. DMRs are ranked by weights from high to low. **(C)** Delta methylation profile, average delta methylation and absolute methylation levels of 225 DMRs with most negative weights in PC4. DMRs are ranked by weights from high to low.

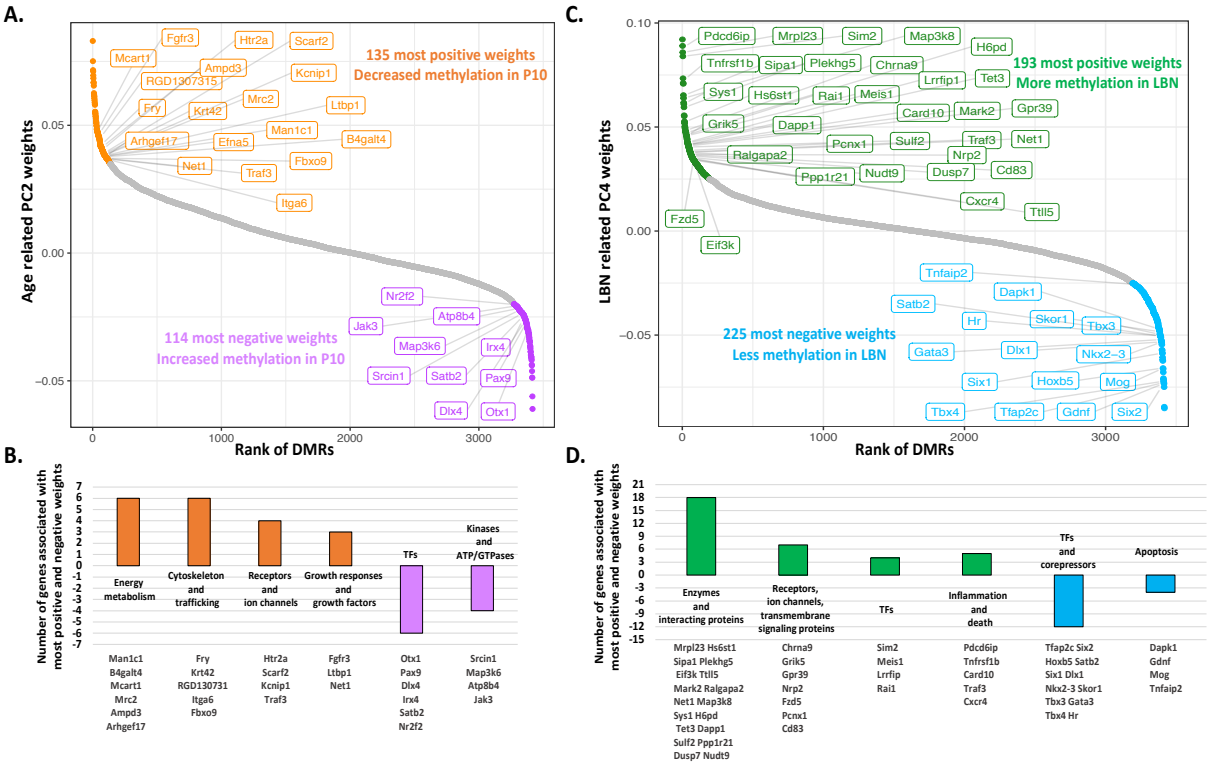

Figure 4. Expected consequences of age- and experience- related differentially methylated regions (DMRs).

(A) Analysis of the PC2 weights that separate by age the P2 and P10 samples. Most positive (orange) and negative (purple) weights are enriched in genes associated with functional categories listed in **(B)**. **(C)** Analysis of PC4 weights that separate individual rats that had experience early-life adversity (LBN) or typical rearing conditions (controls). Most positive (darkgreen) and negative (skyblue) weights are enriched in genes associated with functional categories listed in **(D)**.

Materials and Methods

Animals

Subjects were born to primiparous time-pregnant Sprague-Dawley rat dams (around P75) that were maintained in the quiet animal facility room on a 12 h light/dark cycle with *ad libitum* access to lab chow and water. Parturition was checked daily, and the day of birth was considered postnatal day 0 (P0). Litter size was adjusted 12 per dam on P1, if needed. On P2, pups from several litters were gathered, and 12 pups (6 males and 6 females) were assigned at random to each dam, to obviate the potential confounding effects of genetic variables and of litter size. Each pup was identified by a rapid (<2 minute) foot pad tattooing using animal tattoo ink (Ketchum).

Early-life adversity paradigm

The experimental paradigm involved rearing pups and dams in "impoverished" cages for a week (P2-P9) as described elsewhere [30–32]. Briefly, routine rat cages were fitted with a plastic-coated aluminum mesh platform sitting ~2.5 cm above the cage floor (allowing collection of droppings). Bedding was reduced to only cover cage floor sparsely, and one-half of a single paper towel was provided for nesting material, creating alimited bedding and nesting (LBN) cage. Control dams and their litters resided in standard bedded cages, containing 0.33 cubic feet of cob bedding, which was also used for nest building. For each experiment, pups form several litters were mixed, and then assigned randomly to a control or LBN dam. This procedure minimizes the potential effects of pup genetic background on outcomes. Control and experimental cages were undisturbed during P2–P9, housed in a quiet room with constant temperature and a strong laminar airflow, preventing ammonia accumulation. For technical reasons the study was conducted in two 'batches' (cohorts). These differed solely in the dates at which they were conducted.

Collection of buccal swab from each pup

The first buccal swab was collected from both cheeks of each pup prior to randomization on P2, using Hydraflock swab (Puritan diagnostics, LLC). After an hour's rest with their mother, a second buccal swab was collected, enabling sufficient DNA from each pup. Pups were then randomized to controls or LBN cages. During P3-P9, behaviors of dams in both control and adversity/LBN cages was observed daily, to ascertain the generation of fragmented unpredictable caring patterns by the adverse environment [33,34]. On P10, buccal swabs were collected as described for P2, then all litters were transferred to normal bedded cages.

Isolation and quantification of DNA for making RRBS libraries from Rat Buccal swab

The Buccal swab was placed into the DNA shields™ (Zymo Research) immediately after swabbing. DNA was prepared from the DNA shields solution using the Quick-gDNA™ MiniPrep kit (Zymo Research) following the manufacturer's protocol. The quantity of double stranded DNA was analyzed using Qubit, and RRBS Libraries were prepared from 40 ng of genomic DNA digested with Msp I and then extracted with DNA Clean & Concentrator™-5 kit (Zymo Research). Fragments were ligated to pre-annealed adapters containing 5'-methyl-cytosine instead of cytosine according to Illumina's specified guidelines (www.illumina.com). Adaptor-ligated fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning™ Kit (Zymo Research). Preparative-scale PCR was performed and the resulting products were purified with DNA Clean & Concentrator for sequencing. Amplified RRBS libraries were quantified and qualified by Qubit, Bioanalyzer (Agilent), and Kapa library quant (Kapa systems), and then sequenced on the Illumina NextSeq 500 platform.

RRBS data processing and detection of differentially methylated regions (DMRs)

Adaptor and low quality reads were trimmed and filtered using Trim Galore! 0.4.3 [35] with parameter '--fastqc --stringency 5 --rrbs --length 30 --non_directional'. Reads were aligned to the rat genome (RGSC 6.0/rn6) by using Bismark 0.16.3 [36] with '--non_directional' mode. CpG sites were called by "bismark_methylation_extractor" function from Bismark. Single CpG sites with more than 10 reads coverage were kept for DMR calling. Differential methylation sites (DMSs) were first called using MethyKit (R 3.3.2) [37] between P2 and P10 from the same individual with a false discovery rate (FDR) lower than 0.05. DMSs were shared in at least two individuals in either control or LBN groups were kept and DMSs falling within 100 base pairs were then merged into DMRs.

Calculation of DNA methylation level/percentage and Delta methylation

The methylation percentage/level was calculated as the ratio of the methylated read counts over the sum of both methylated and unmethylated read counts for a single CpG site or across CpGs for a region. The delta methylation was calculated using the log2 transformation of the ratio of methylation level in the P10 sample and the methylation level in the P2 sample, defined as

$\log_2$(P10/P2). Increased methylation in P10 is shown as a positive value while decreased methylation in P10 is shown as a negative value.

Principal component analysis (PCA) and K-Means clustering

Before PCA analysis, DNA methylation level of DMRs are batch corrected by using *removeBatchEffect* function from *limma* (R package) with setting cohorts as batches. PCA analysis was performed on these batch corrected DMRs by using *IncrementalPCA* function from scikit-learn [38] using python 2 for both Figure 2 and 3. The value of k was set to 10 for the k-means clustering based on a preliminary hierarchical clustering analysis. A DNA methylation heatmap was generated with heatmap.2 function in R 3.5.0 and a delta methylation heatmap was generated using Java TreeView [39].

Gene analysis

Genes associated with DMRs were identified using Homer 4.7 [40]. For subsequent analyses, genes were kept if (1) CpGs were located within 20kb of TSS in intergenic, promoter-TSS and TTS positions; (2) CpGs were located within gene exons or introns. Gene ontology analysis was performed using Metascape [41] using the hypergeometric test with corrected P-value lower than 0.05.

Data Access

Reads and processed data from RRBS assays have been submitted to the GEO data repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE119640.

Supplementary Information

A.

Cohort 1

P2	Mapped reads (million)	Efficiency	Ratio of Methylated C in CpG	P10	Mapped reads (million)	Efficiency	Ratio of Methylated C in CpG
P2C3	13	43.30%	24.4%	P10C3	23	46.00%	28.1%
P2C4	9	36.1%	25.0%	P10C4	19	55.1%	24.5%
P2C6	12	51.30%	24.3%	P10C6	17	44.40%	24.2%
P2C7	22	59.50%	24.3%	P10C7	26	52.00%	25.9%
P2C9	18	41.10%	24.4%	P10C9	26	53.70%	28.1%
P2C12	20	49.20%	22.8%	P10C12	24	52.20%	27.8%
P2LBN2	15	39.90%	22.3%	P10LBN2	11	45.90%	27.3%
P2LBN3	18	43.20%	24.4%	P10LBN3	30	58.90%	26.9%
P2LBN4	23	48.80%	22.0%	P10LBN4	28	51.00%	29.0%
P2LBN9	12	46.40%	24.0%	P10LBN9	24	50.40%	27.6%
P2LBN12	15	45.40%	20.2%	P10LBN12	26	56.40%	27.8%

Individual	Detected CpGs in both P2 and P10	Significant DMRs
C3	418860	870
C4	358726	4664
C6	404260	4384
C7	521418	1959
C9	514738	2679
C12	496953	2318
LBN2	415216	5540
LBN3	482076	6345
LBN4	539139	629
LBN9	418466	1851
LBN12	443196	1963

B.

Cohort 2

P2	Mapped reads (million)	Efficiency	Ratio of Methylated C in CpG	P10	Mapped reads (million)	Efficiency	Ratio of Methylated C in CpG
P2C1	21	46.0%	21.6%	P10C1	22	42.5%	17.0%
P2C5	20	47.4%	20.5%	P10C5	20	45.6%	21.2%
P2C8	17	60.4%	21.5%	P10C8	22	49.2%	18.6%
P2C11	19	55.7%	21.1%	P10C11	27	58.1%	17.2%
P2LBN2	28	57.8%	26.0%	P10LBN2	30	61.9%	19.7%
P2LBN4	21	49.2%	21.4%	P10LBN4	20	42.0%	20.3%
P2LBN9	21	45.8%	21.0%	P10LBN9	22	54.2%	17.0%
P2LBN11	23	47.2%	19.5%	P10LBN11	20	50.8%	18.3%

Individual	Detected CpGs in both P2 and P10	Significant DMRs
C1	510525	3073
C5	505100	1879
C8	508787	1278
C11	525733	7040
LBN2	584480	1064
LBN4	498882	2566
LBN9	521111	1190
LBN11	504507	2745

Figure S1. Quality control of RRBS data.
Quality control matrix for **(A)** cohort1 and **(B)** cohort2**,** including the number of uniquely mapped reads, mapping efficiency, ratio of methylated C in CpG and significant DMRs calling for each individual.

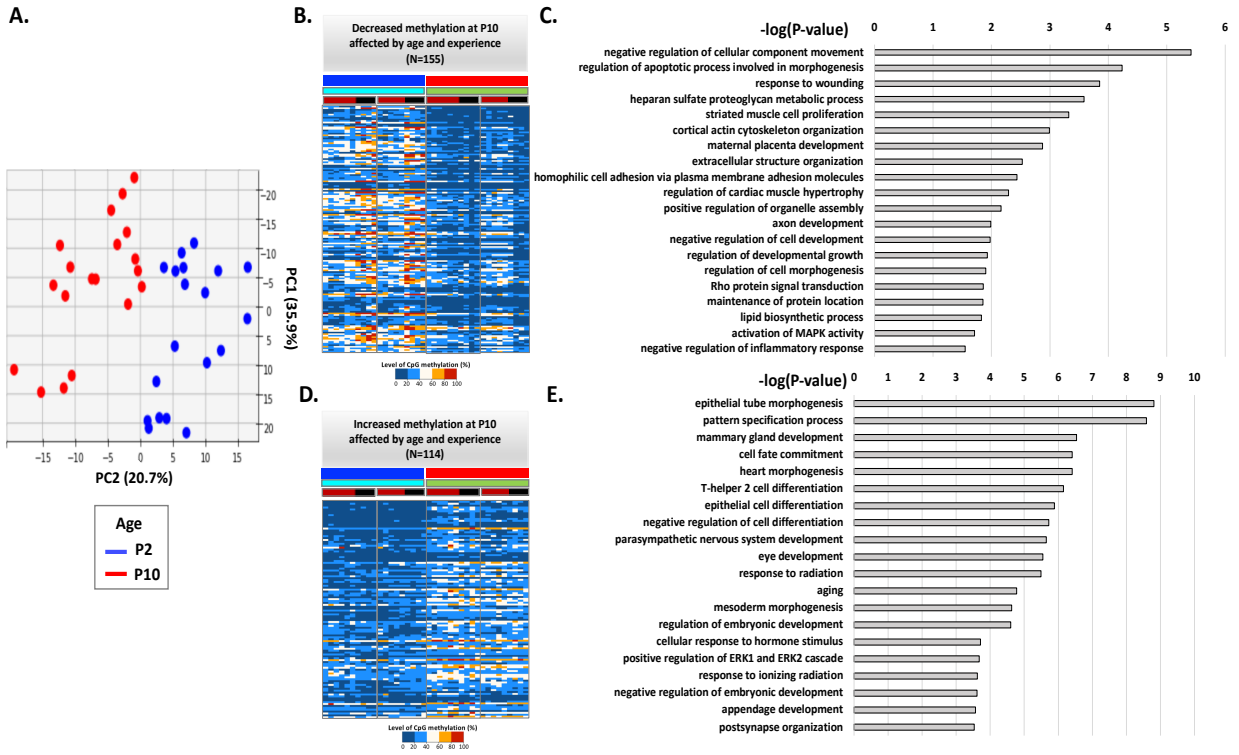

Figure S2. Annotation of age-related DMRs in controls and LBNs.
(A) Top view of PCA in Figure 2B. PC2 is the major component that separates P2 and P10 individuals. **(B)** Methylation level of 155 DMRs with most positive weights in PC2, showing decreased methylation at P10. **(C)** Gene ontology terms enriched in the genes associated with these 155 DMRs. **(D)** Methylation level of 114 DMRs with most negative weights in PC2, showing increased methylation at P10. **(E)** Gene ontology terms enriched in the genes associated with these 114 DMRs.

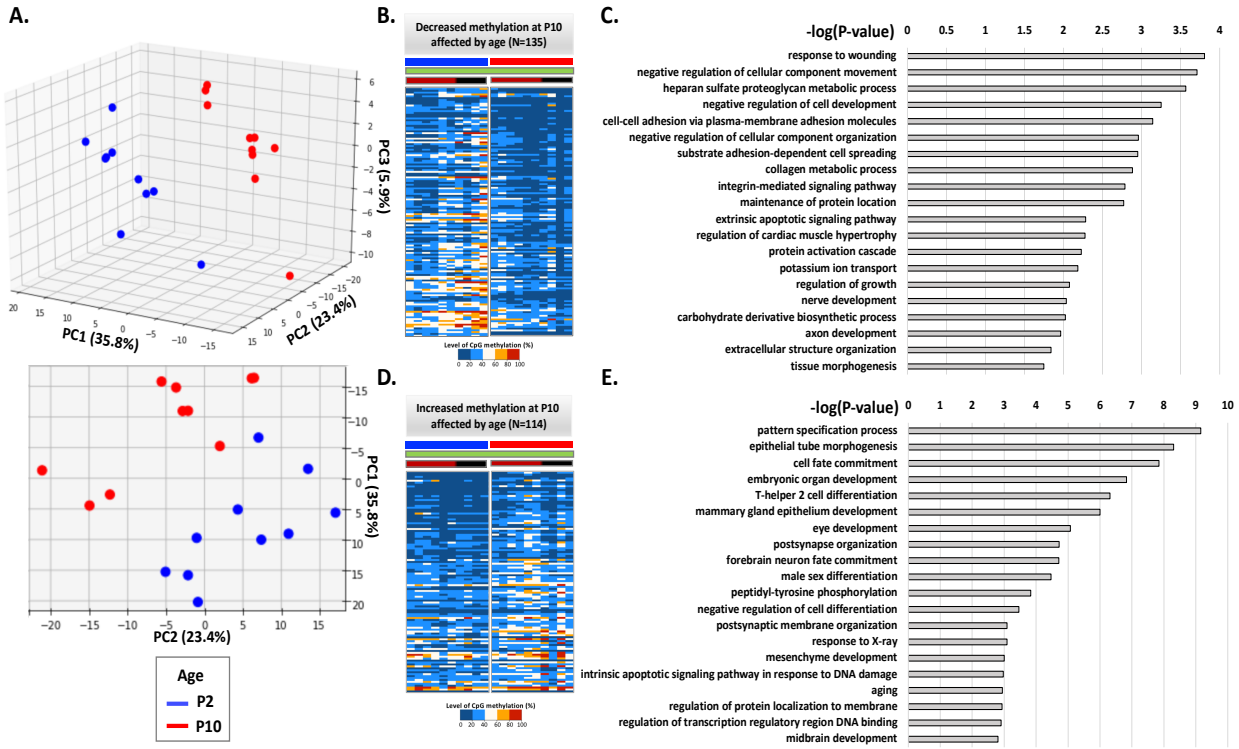

Figure S3. Annotation of age-related DMRs only in controls.
(A) Side view and top view of PCA only with control individuals. PC2 separates P2 and P10 individuals. **(B)** Methylation level of 135 DMRs with most positive weights in PC2, showing decreased methylation at P10. **(C)** Gene ontology terms enriched in the genes associated with these 135 DMRs. **(D)** Methylation level of 114 DMRs with most negative weights in PC2, showing increased methylation at P10. **(E)** Gene ontology terms enriched in the genes associated with these 114 DMRs.

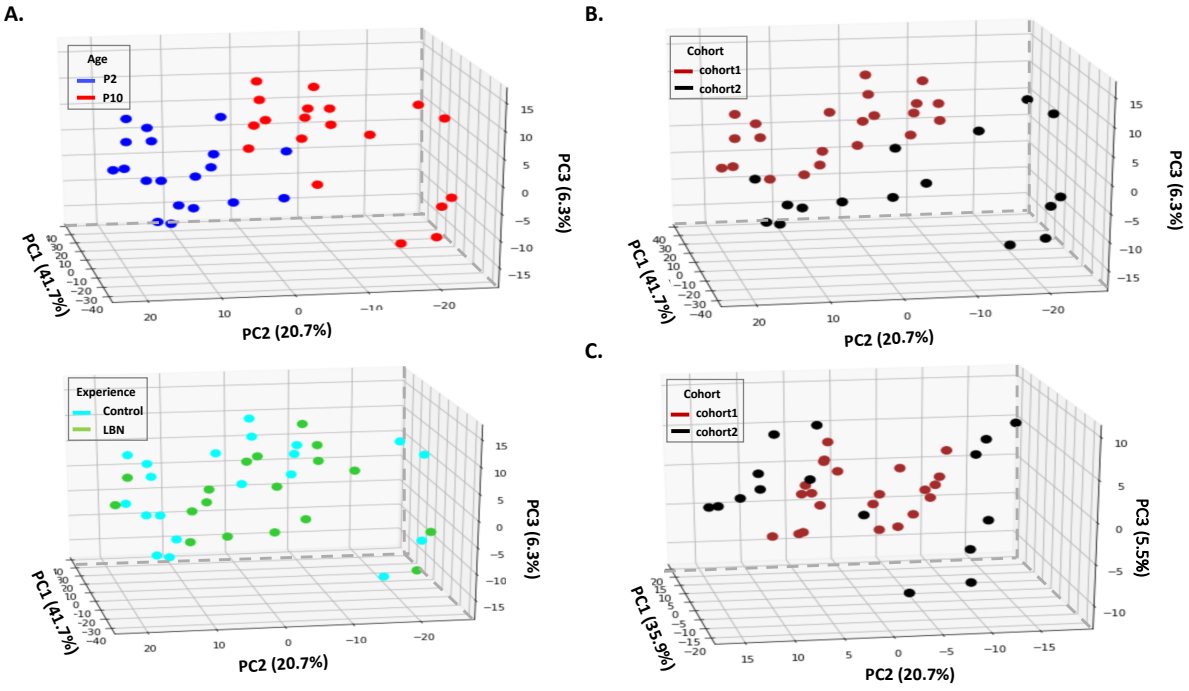

Figure S4. Examination of potential batch effects between two cohorts.
(A) PCA analysis on the methylation level of 3417 DMRs before corrected the batch effects by cohorts. Individuals can still be separated by age on PC2 but cannot be separated by their experiences. **(B)** PC1 is the major component that separates individuals by cohorts before doing batch correction. **(C)** PCA analysis on the methylation level of DMRs after corrected by cohorts, same as Figure 2B and C. Individuals cannot be separated by cohorts.

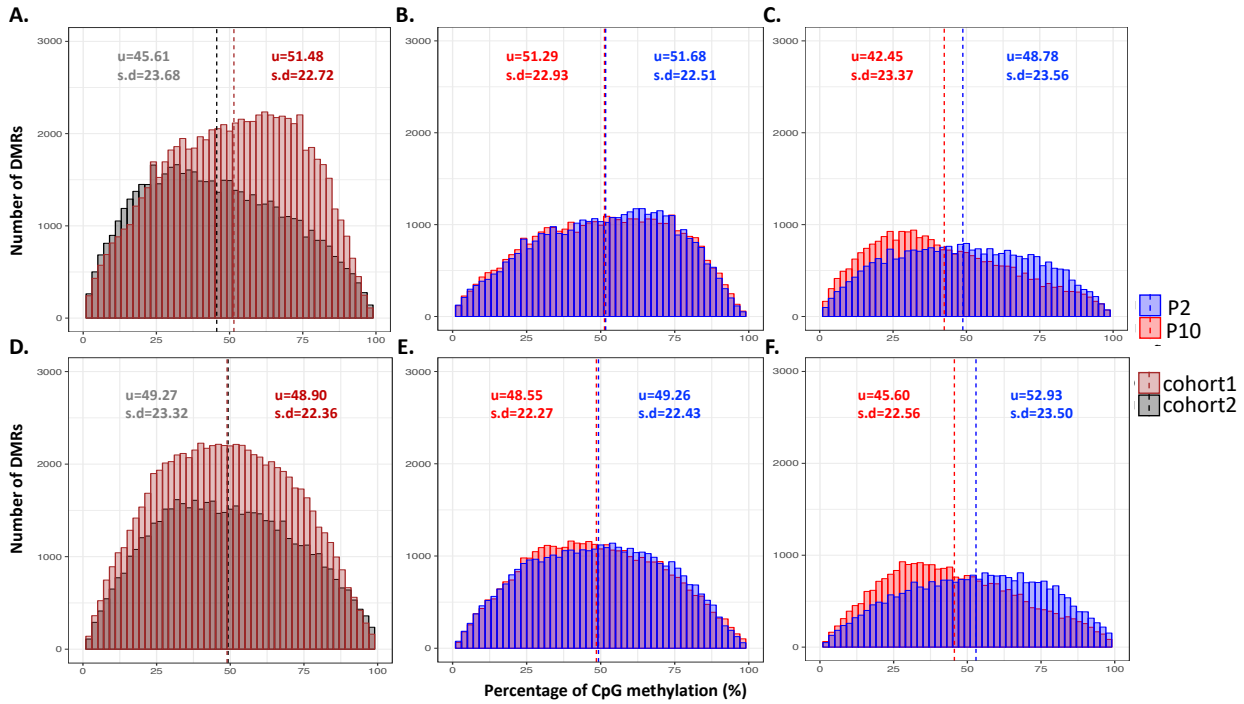

Figure S5. Distribution of methylation level on DMRs across individuals.
(A) Histogram of methylation level on 3417 DMRs across 19 individuals from two cohorts before batch correction by cohorts ($p<2.2x10^{-16}$, Mann-Whitney U Test). For each DMR, each individual has one methylation level for P2 and one for P10. 11 individuals are in cohort1 and 8 individuals are in cohort2.
(B) Histogram of methylation level on 3417 DMRs on cohort1 for P2 and P10 separately (before correction by cohorts).
(C) Histogram of methylation level on 3417 DMRs on cohort2 for P2 and P10 separately (before correction by cohorts).
(D) Histogram of methylation level on 3417 DMRs across 19 individuals from two cohorts after batch correction by cohorts (p=0.2197, Mann-Whitney U Test). For each DMR, each individual has one methylation level for P2 and one for P10. 11 individuals are in cohort1 and 8 individuals are in cohort2.
(E) Histogram of methylation level on 3417 DMRs on cohort1 for P2 and P10 separately (after correction by cohorts).
(F) Histogram of methylation level on 3417 DMRs on cohort2 for P2 and P10 separately (after correction by cohorts).
u represents mean value; s.d represent standard deviation.

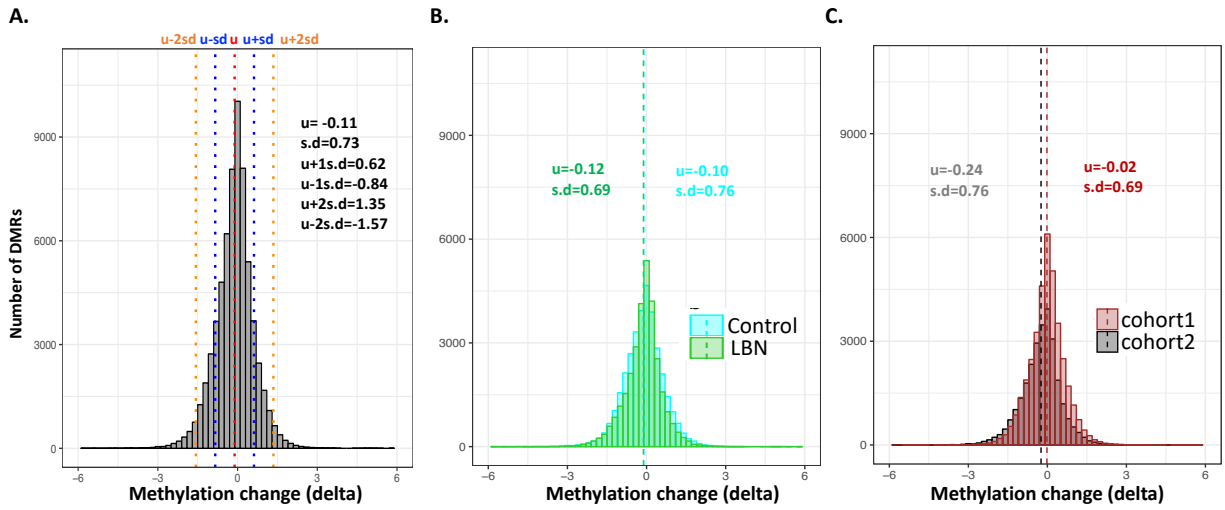

Figure S6. Distribution of delta methylation on DMRs across individuals.
(A) Histogram of delta methylation on 3417 DMRs across 19 individuals from two cohorts.
(B) Histogram of delta methylation on 3417 DMRs, labeled by control and LBN separately.
(C) Histogram of delta methylation on 3417 DMRs, labeled by two cohorts separately.
u represents mean value; s.d represent standard deviation.

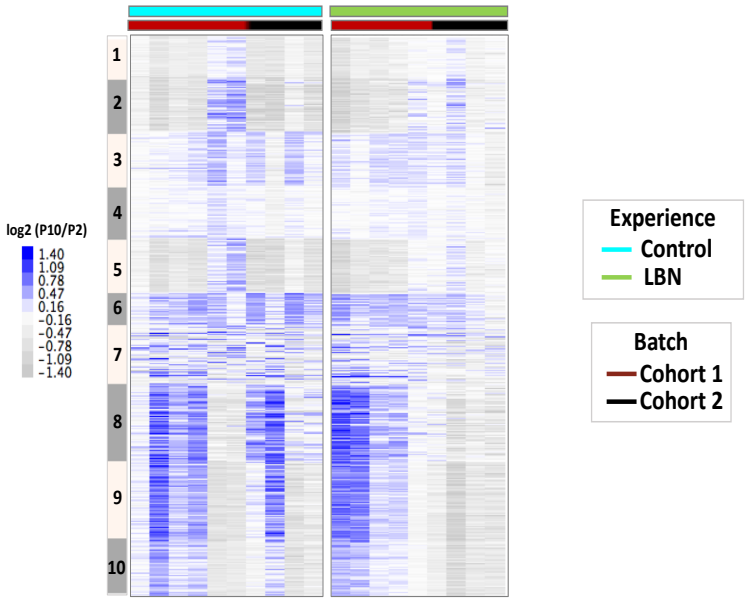

Figure S7. Delta methylation changes in controls and LBNs.
Heatmap of delta methylation (log2(P10/P2)) profiles on 3417 DMRs in control and LBN individuals. DMRs are in the same order as in Figure 2A.

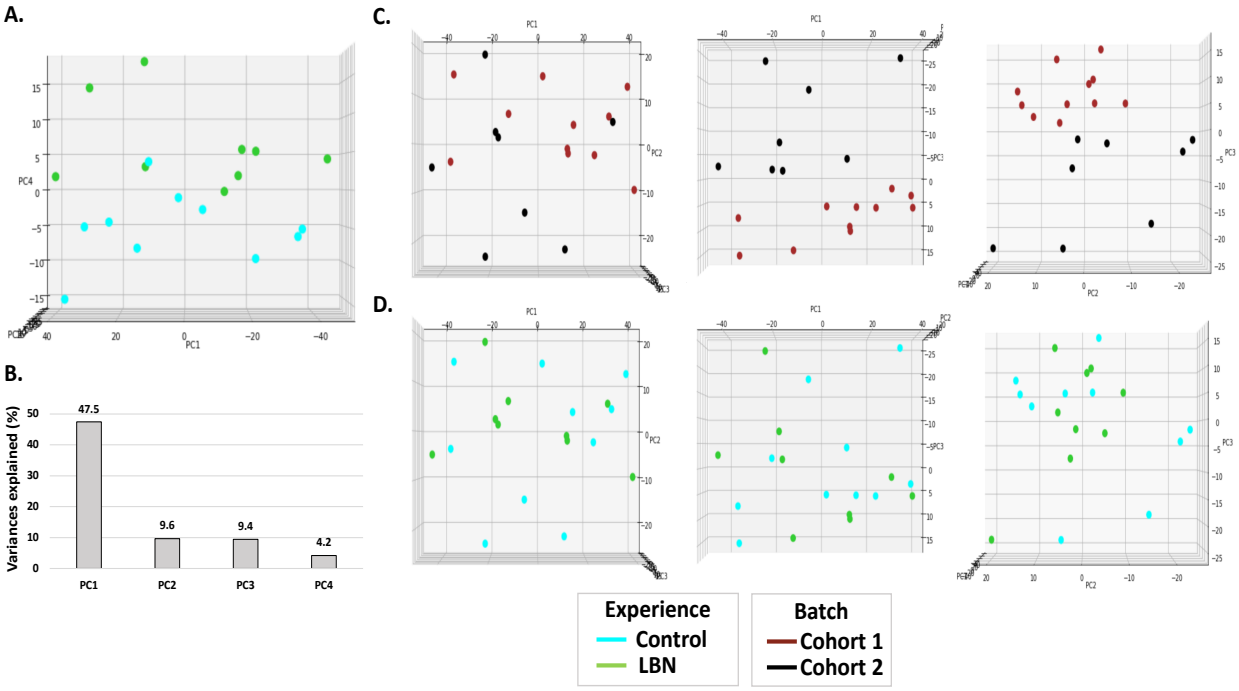

Figure S8. Supplementary information of delta methylation PCA.
(A) Top view of PCA in Figure 3A. PC4 is the major component that separates control and LBN individuals. **(B)** Variances of first 4 components in delta methylation PCA analysis. Top view of delta methylation PCA in PC1 vs PC2, PC1 vs PC3 and PC2 vs PC3 dimensions. Individuals are labeled by **(C)** cohorts and **(D)** experiences.

Supplemental Table S1. Annotation of age-related DMRs associated with most positive and negative weights of PC2 in Figure S3A. Methylation levels are listed for each DMRs across all individuals.

Supplemental Table S2. Annotation of experience-related DMRs associated with most positive and negative weights of PC4 in Figure 3A. Delta methylation changes are listed for each DMRs across all individuals.

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Author contributions

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Conflict of Interest Statement

The authors declare that they have no conflict of interest

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