Early life adversity alters normal sex-dependent developmental dynamics of DNA methylation

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Abstract

Studies in rodents, nonhuman primates, and humans suggest that epigenetic processes mediate between early life experiences and adult phenotype. However, the normal evolution of epigenetic programs during child development, the effect of sex, and the impact of early life adversity on these trajectories are not well understood. This study mapped the genome-wide DNA methylation changes in CD3+ T lymphocytes from rhesus monkeys from postnatal day 14 through 2 years of age in both males and females and determined the impact of maternal deprivation on the DNA methylation profile. We show here that DNA methylation profiles evolve from birth to adolescence and are sex dependent. DNA methylation changes accompany imposed weaning, attenuating the difference between males and females. Maternal separation at birth alters the normal evolution of DNA methylation profiles and targets genes that are also affected by a later stage maternal separation, that is, weaning. Our results suggest that early life events dynamically interfere with the normal developmental evolution of the DNA methylation profile and that these changes are highly effected by sex.

It is widely accepted that early life social experiences play an important role in defining lifelong phenotypes, including increased risk for developing psychiatric disorders such as anxiety and major depression (Heim & Nemeroff, 2001; Kaufman, Plotsky, Nemeroff, & Charney, 2000; McEwen, 2000) as well as chronic diseases during adulthood (Power et al., 2007). During the last decade, an increasing body of data has emerged that is consistent with the idea that epigenetic mechanisms are mediating between early life experiences and adult phenotypes.

Epigenetic processes are responsible for conferring upon identical genes different levels of activities in different tissues during embryogenesis (Razin & Cedar, 1993). This could explain how different tissues express widely divergent phenotypes although they carry almost identical genomes. Epigenetic mechanisms include modifications of histone proteins (Strahl & Allis, 2000), noncoding regulatory RNAs such as

microRNA (Bartel, 2004) as well as chemical modification of the DNA molecule itself by methylation of cytosine (Hotchkiss, 1948; Wyatt, 1950) or adenine residues (Theil & Zamenhof, 1963; Wu et al., 2016), and further modification of the methyl group on DNA by hydroxylation (Kriaucionis & Heintz, 2009). DNA methylation is part of the chemical entity of DNA, which makes it different from all other epigenetic mechanisms that involve proteins and RNAs that interact with DNA. Hence, the DNA molecule itself has two identities, the inherited DNA sequence or the ancestral identity and the DNA methylation profile that is programmed during gestation and confers upon DNA a cellular identity. DNA methylation in critical positions in genes such as promoters or enhancers could silence gene expression (Levine, Cantoni, & Razin, 1991). DNA methylation in other regions such as gene bodies might activate gene expression (Hellman & Chess, 2007).

Epigenetic modifications are enzymatically catalyzed and are regulated by cellular signaling pathways (Szyf, Weaver, & Meaney, 2007; Weaver et al., 2014), and are therefore well positioned to respond to external cues in addition to developmental programs. It was therefore proposed that DNA methylation could respond to experiences and confer upon DNA an experiential identity in addition to the tissue-specific identity (Szyf, 2012). DNA methylation is proposed to mediate between experience and the phenotype by altering gene activity (Szyf, 2012).

Evidence that early life experience causes epigenetic changes came from studies of maternal care in rats. In these studies, differences in maternal care triggered differences in DNA methylation in the offspring hippocampus that persisted into adulthood, which changed expression of the glucocorticoid receptor in the hippocampus, and resulted in differences

of stress response (Weaver et al., 2004). These studies provide a paradigm for experience-mediated epigenetic programming whereby experience triggers cellular pathways that cause a change in DNA methylation in a candidate gene. This DNA methylation alteration remains a genomic memory of the experience and determines gene activity throughout life by precipitating a stable phenotype (Meaney & Szyf, 2005).

Other studies that focused on candidate genes in specific brain regions further confirmed DNA methylation alterations in response to early life behavioral experiences in both rats and humans. There is an association of childhood abuse in humans with DNA methylation levels in the promoters of ribosomal RNA genes (McGowan et al., 2008) and exon *1f* of the glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1 [*NR3C1*]) gene promoter (McGowan et al., 2009). Exposure of infant rats to stressed caretakers produced persistent changes in DNA methylation of the brain derived neurotrophic factor (*BDNF*) gene promoter in the adult prefrontal cortex (Roth, Lubin, Funk, & Sweatt, 2009). Early-life stress in mice caused sustained DNA hypomethylation in the *arginine vasopressin* gene (Murgatroyd et al., 2009).

Changes in DNA methylation triggered by behavioral experience are not limited to a few candidate genes or particular brain regions. Broad changes in DNA methylation were observed in the hippocampi of adult rats who were exposed to low maternal care (McGowan et al., 2011) or in humans who were abused as children (Suderman et al., 2012). Studies of patients with posttraumatic stress disorder revealed a stress-system related Gene × Environment interaction: DNA methylation changes have been shown in white blood cells triggered by child abuse in combination with a risk allele of the FK506 binding protein 5 gene (*FKBP5*; coding for FK506 BP 51 kDa, i.e., FKBP51), which is an important factor in the intracellular negative feedback loop of glucocorticoids (Klengel et al., 2013). Genome-wide changes in DNA methylation were observed in blood of abused children, children who were exposed to trauma as children and then developed posttraumatic stress disorder (Mehta et al., 2013), and children who were exposed to trauma and exhibited differences in cortisol reactivity later in life (Houtepen et al., 2016). The observed "system-wide" and "genome-wide" nature of the response to DNA methylation is consistent with the phenotypic consequences of early child experience that involve both physical (Power et al., 2007) and mental (Heim & Nemeroff, 2001; Kaufman et al., 2000; McEwen, 2000) health.

It is conceivable that the DNA methylation signature of child adversity that is found in peripheral tissues is mediating the system-wide physiological effects of social experience and might not reflect the alterations in gene programming that take place in the brain (Szyf, Tang, Hill, & Musci, 2016). However, we find DNA methylation changes in immune cells that associate with particular behavioral phenotypes and alterations in brain function rather than just physical phenotypes. These findings run counter to the common understanding that the effects of particular alterations in epigenetic processes are limited to that tissue (Razin & Szyf, 1984).

Nevertheless, changes in methylation of genes such as the serotonin transporter (solute carrier family C6, member 4 [*SLC6A4*]) were associated with differences in brain imaging of serotonin synthesis (Wang et al., 2012), hippocampal gray matter volume (Dannlowski et al., 2014), and processing of emotional stimuli (Dannlowski et al., 2014). These data suggest that DNA methylation in periphery could teach us in certain instances not only about the physical correlates of the response to behavioral experiences such as the effects on metabolic and immune systems but also about changes in brain-related phenotypes.

This important concept that DNA methylation in the periphery could serve as surrogate markers for gene programming in the brain needs to be confirmed by additional data and a better mechanistic understanding of the relationship between DNA methylation alterations in peripheral immune cells and gene expression in the brain. The plausibility that DNA methylation alterations in periphery are informative for gene expression programming in the brain has important practical implications for studying behavioral epigenetics in living humans, particularly longitudinal studies as well as for the clinical utility of epigenetic markers.

One major limitation of studies in behavioral epigenetics is that most of our results in humans come from association studies, and it is difficult to dissociate the epigenetic changes from genetic causes. Moreover, it is difficult to demonstrate causation between DNA methylation changes and the phenotype because most studies examine associations between DNA methylation and already established phenotypes. In contrast with inborn germline genetic changes that precede the phenotype, DNA methylation alterations could be a consequence rather than a cause of the phenotype. Establishing a temporal relationship between the DNA alteration and the emergence of phenotype is critical; however, most DNA methylation association studies lack a longitudinal component. This is particularly true for the majority of studies that examine the effect of early life adversity years after the early life event, casting doubt on the true link between the exposure and the DNA methylation alteration.

Randomized stress caused by natural disaster allows for a quasi-experimental examination of the causal relationship between "experience" and DNA methylation alterations. Examination of DNA methylation alterations in peripheral T cell DNA of adolescents who were exposed in utero to the Quebec ice storm of 1998 revealed changes in DNA methylation in CD3+ lymphocytes; DNA methylation levels of differentially methylated CG sites correlated with the levels of objective stress of their mothers during pregnancy (Cao-Lei et al., 2014). This study provided the first empirical evidence in humans that prenatal stress could trigger DNA methylation changes that are detectable in adolescent peripheral immune cells. It is interesting that mediation analyses using methylation data, objective stress during pregnancy, and phenotype during adolescence revealed mediation effects of DNA methylation on body mass index, supporting a causal role for DNA methylation in the emerging phenotype (Cao-

Lei et al., 2015). However, these DNA methylation marks were measured 15 years after the initial exposure, and it is unknown whether they were triggered by the exposure or by other downstream experiences later in life, or whether these differences in DNA methylation were also present earlier in life when the exposure to early life stress has occurred.

Nonhuman primates provide a superior model to address some of these critical limitations in behavioral epigenetics. In the model examined here, rhesus macaque monkeys are randomly assigned at birth to differential rearing conditions, reared by either their mother in a social setting or by an inanimate, cloth-covered surrogate with continual exposure to peers. This randomized design that is infeasible in humans enables testing causality between early life adversity and epigenetic changes. The surrogate rearing condition serves to isolate the effects of maternal care for downstream events, and models relevant early life stressors in humans. In nonhuman primate models, maternal deprivation with peer-to-peer social contact disrupts the mother–infant relationship and leads to emotional and social disturbances and behavioral abnormalities, such as motor stereotypies (Barr et al., 2003; Champoux et al., 2002; Suomi, 1991). Peer-reared macaques develop inadequate social skills in adolescence, including increased anxious and aggressive behavior as well as increased chronic cortisol concentrations (Barr et al., 2003; Dettmer, Novak, Suomi, & Meyer, 2012). As adults, they show increased voluntary alcohol consumption and typically rank at the bottom of the social dominance hierarchy (Barr et al., 2003; Suomi, 1991). We previously examined genome-wide promoter methylation profiles from isolated CD3+ lymphocytes and from the prefrontal cortex of adult male rhesus macaques subjected to either maternal or surrogate rearing conditions after birth. Using the method of methylated DNA immunoprecipitation (MeDIP), we delineated broad DNA methylation changes in the brain as well as in peripheral T cells (Provencal et al., 2012). However, because adult DNA methylation profiles were examined in this study, the temporal relationship of these profiles to maternal deprivation was unknown.

The first example of epigenetic programming by maternal behavior was shown at the *NR3C1* gene that was epigenetically programmed in neonates, and this epigenetic program was "fixed" and remained as a "genomic memory" of the early life exposure into adulthood (Weaver et al., 2004). It is unclear whether this is the case for all epigenetic alterations triggered by early life experience, nor is it known whether DNA methylation profiles remain stable through normal postnatal development. An alternative prospect is that DNA methylation patterns evolve postnatally and that early life experiences have a dynamic effect on the normal evolution of the DNA methylation pattern, altering the trajectories of DNA methylation rather than causing a fixed DNA methylation change that remains the same into adulthood. Early life experience might cause a dynamic shift in developmentally regulated DNA methylation trajectories rather than a fixed DNA methylation change. Such differences in DNA methylation from controls might become detectable only later in life.

In this paper we delineate the normal evolution of DNA methylation in rhesus macaques from 14 days after birth up to early adolescence (2 years) in both males and females and examine the effect of maternal deprivation on the evolution of the DNA methylation pattern. Our data are consistent with the hypothesis that early life maternal deprivation dynamically intervenes with the normal sex-dependent evolution of DNA methylation profiles during postnatal development.

Methods

Animals and rearing procedures

Samples were obtained from both male and female rhesus monkeys (*Macaca mulatta*), aged 2 weeks (Day 14 [D14]) to 30 months, that were born between 2009 and 2011 and housed at the Laboratory of Comparative Ethology, part of the National Institute of Child Health and Human Development, at the National Institutes of Health Animal Center (Poolesville, MD). The maternal deprivation experiments have been described previously in detail (Conti et al., 2012). The monkeys were randomly divided into mother-reared (MR) and surrogate peer-reared (SPR) groups at birth (these were raised individually in a nursery in the first month of their life). Until imposed weaning (7–8 months of age), the MR monkeys lived with their mothers in large social groups of 8–10 adult females, 1 adult male, and 3–5 same-aged peers; the SPR monkeys lived in individual cages with an inanimate surrogate mother, and continual visual, tactile, and olfactory access to same-aged peers. SPR monkeys had 2 hr of daily socialization periods with age-matched peers. After imposed weaning, all monkeys (MR and SPR) were relocated from their living conditions and placed in a mixed social group in a different building where they lived up through 3 years of age. All environmental conditions, procedures, and handling of animals were in strict compliance with the requirements of the National Institute of Child Health and Human Development Animal Care and Use Committee, and all experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Preparation of CD3+ T lymphocyte samples

The first blood samples were obtained from monkeys aged 14–30 days old (D14 samples; MR males, $n = 6$; SPR males $n = 5$; MR females, $n = 3$; SPR females $n = 3$). The first 6 months of nonhuman primate development is roughly equivalent to the first 2 years of human life, which is a critical period for appropriate sociocognitive development; therefore, the second sample was obtained at age 6–7 months (before weaning [BW]; MR males, $n = 6$; SPR males $n = 7$; MR females $n = 3$; SPR females $n = 4$). At approximately 8 months of age, MR and SPR monkeys were placed in a new, large social group. Because this period is stressful for the young monkeys (Dettmer et al., 2012), the third sampling was timed after weaning (AW) at 9–10 months of age (MR males, $n = 5$; SPR

males $n = 7$; MR females $n = 4$; SPR females $n = 4$). The fourth time point was after 2 years (2y samples), between 26 and 30 months (MR males, $n = 6$; SPR males $n = 4$; MR females $n = 2$; SPR females $n = 2$).

Separation of CD3+ T cells from monkey peripheral blood was performed as previously described (Provencal et al., 2012). In brief, peripheral blood was drawn into EDTA-coated tubes. Peripheral blood mononuclear cells were isolated through centrifugation with Ficoll-Paque (GE Healthcare), and T cells were isolated using CD3+ Dynabeads (Life Technologies, Burlington, ON, Canada).

The CD3+ T cell DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The double-stranded DNA concentration was measured using the Qubit system (Life Technologies). The D14 samples were pooled DNA (male and female separately) from six MR and six SPR monkeys sampled twice (at postnatal Days 14 and 30) because of low DNA yield in individual samples. The DNA pooling procedure was applied for all time points, using the same amount of double-stranded DNA from each individual sample, creating four different groups at the actual developmental stage: male MR (control), male SPR (stress), female MR (control), and female SPR (stress).

Analysis of genome-wide promoter DNA methylation

Genome-wide DNA methylation analysis was performed using the MeDIP protocol as previously described (Provencal et al., 2012). Briefly, 2 μg of genomic DNA per pooled DNA sample were sonicated, and methylated DNA was immunoprecipitated using an anti-5-methyl-cytosine antibody (Eurogentec, Fremont, CA). The input and bound fractions were amplified using a Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO) and were labeled for microarray hybridization with Cy3-dUTP and Cy5-dUTP, respectively, using a CGH Enzymatic Labeling Kit (Agilent Technologies, Mississauga, ON, Canada) in accordance with the manufacturer's instructions. Custom designed 400K promoter tiling arrays were used (Agilent Technologies). Microarray probe sequences were selected to tile at 100 base pair spacing all gene promoter regions defined as the genomic interval from −1800 base pairs upstream to 400 base pairs downstream of each transcription start site as defined for the *Macaca mulatta* genome by the Ensembl database (Version 64.10). All steps of the hybridization, washing, scanning, and feature extraction were performed following the Agilent protocols for chip-on-chip analysis (MeDIP).

After microarray scanning, probe intensities were extracted from scan images using Agilent's Feature Extraction 10.5 Image Analysis Software. The extracted intensities were then analyzed using the R software environment for statistical computing. 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, Irizarry, Astrand, & Speed, 2003) under the assumption that all samples have identical overall methylation levels. Linear models implemented in the *limma*

package (Smyth, 2005) of Bioconductor were used to compute a modified t statistic from the normalized intensities of the probes across all samples between the two groups.

To analyze methylation differences in CD3+ cell samples, we calculated the false discovery rate as previously published. An individual probe was called differentially methylated if the significance of its t statistic was at most 0.01 (uncorrected for multiple testing) and the associated difference of means between the groups was at least 0.5. Correlation between technical replicates was modeled as a random effect using the "block" variable. Significance was determined using the Wilcoxon rank-sum test comparing t statistics of these probes against those of all the probes on the microarray. The resulting p values for each gene were then corrected for multiple testing by calculating their false discovery rate. DNA methylation differences of the gene promoters were calculated by averaging the methylation differences of significantly affected probes (false discovery rate $q < 0.2$) of each gene promoter region as described (Provencal et al., 2012).

For biological function analyses, selected genes were overlaid on the global molecular network developed from information contained in the Ingenuity Pathway knowledge base (http://www.ingenuity.com). The significance of the association between the data sets and biological functions or canonical pathway is scored using a p value calculation (right-tailed Fisher exact test).

Validation of DNA methylation analyses

Gene-specific real-time polymerase chain reaction (PCR) validations of MeDIP microarrays were performed on the amplified input and bound fractions using 20 ng of DNA per reaction. SYBR green quantitative PCR was performed using the LightCycler® 480 system (Software 3.5, Roche Molecular Biochemicals). To determine the relative DNA enrichment, the 2–ΔΔCt method was used.

Gene region-specific DNA methylation analyses were performed by pyrosequencing using PyroMark Q24 or Q96 (Qiagen, Venlo, Limburg, The Netherlands) at the CFI-Imaging and Molecular Biology Platform in the Department of Pharmacology and Therapeutics, McGill University. For bisulfite conversion we used 500 ng of genomic DNA with an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA), and PCR amplification was carried out using 20 ng of converted DNA with the EpiMark Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA). The methylation percentage at each CpG site was analyzed using PyroMark Q24 or CpG 1.0.11 software (Qiagen). The PCR amplification efficiency was checked with control calibration samples at each run, and in case of biased amplification, the hyperbolic curve fit correction was applied as proposed by Moskalev et al. (2011; using the formula of their equation 3).

Graphpad 5 software (La Jolla, CA) was used to perform statistical analyses. The student's unpaired t test was used and the α level was set at 0.05. When variances were significantly different between groups, the Welch correction was applied.

Results

Evolution of the T cell DNA methylation profile during postnatal development

Blood samples were obtained from monkeys at four time points in life: 14–30 days after birth, at 6 months just prior to the imposed weaning in both rearing conditions, at 9–10 months after the imposed weaning and introduction to new social environment, and at 2 years when the monkeys entered puberty. CD3+ T cells were isolated, DNA was prepared, and the state of DNA methylation in all known gene promoters of the rhesus monkey genome were determined using MeDIP followed by hybridization to high-density oligonucleotide arrays as described in Provencal et al. (2012). The results presented in Figure 1a illustrate the dramatic changes in DNA methylation in T cells of MR (control) animals between Days 14–30 and 6 months in both males and females. These changes affect wide swaths of the genome (Figure 1b, D14-BW changes). It is interesting that there is a reversal in the direction of changes in DNA methylation (shifting from hypo- to hypermethylation) during the weaning period (see online only Supplementary Figure S.1: BW-AW changes compared to D14-BW changes). In addition, the changes in DNA methylation profiles that develop prior to weaning are reduced throughout the developmental stages (Figure 1b non-shaded parts of the columns), but new differences also appear (Figure 1b shaded parts of the columns). The pattern is further modified after the imposed weaning period until 2 years of age (Figure 1a D14-AW and 1b D14-2y changes). Hundreds of genes return to the methylation state observed at Days 14–30 while new genes become either hypo- or hypermethylated (see online only Supplementary Figure S.1, AW-2y changes compared to D14-2y changes). A group of 1,668 promoters are changed between Days 14–30 and 6 months, and their changes are fixed thereafter (see online only Supplementary Table S.1). An inspection of the top pathways of these differentially methylated genes, which do not undergo further changes later in development (and are common to males and females), include genes involved in pluripotency and stem cell properties as well as signaling pathways, suggesting a reprogramming of primary regulatory genes during early postnatal development (Figure 1c).

Sex differences in trajectories of DNA methylation

The results presented in Figure 1 show that both males and females show dynamic changes in DNA methylation during development, but there are differences in number of hypomethylated genes, and although many common genes are affected, differences between sexes are noted as well (Figure 1b). We analyzed the scope of differences between males and females during normal development, which are presented in Figure 2. The heat map in Figure 2a reveals broad differences in DNA methylation in both directions (hypomethylation in blue and hypermethylation in red) between the males and females that

emerge already in the first month. It is important that the changes in DNA methylation are not limited to the X chromosome as expected as one copy of X is inactivated in females by DNA methylation (Mohandas, Sparkes, & Shapiro, 1981); the vast majority of differences in DNA methylation occur in autosomal chromosomes (Figure 2b). These differences in DNA methylation between the sexes are dynamic throughout development from Days 14–30 to 2 years, with broad changes in DNA methylation profiles between males and females at different stages of development (Figure 2c). The difference between the sexes is dramatically reduced after imposed weaning (AW; Figure 2a, c). The state of methylation in both sexes was inversely correlated before (BW) and after (AW) imposed weaning, suggesting that this process involves a reversal of the methylation state prior to imposed weaning (Figure 2d). After weaning the differences in DNA methylation profiles between males and females are reduced; imposed weaning involves opposite changes in DNA methylation in males and females (Figure 2e). Genes that are hypomethylated in males relative to females before imposed weaning (BW) become hypermethylated in males and hypomethylated in females afterward (AW), and genes that are hypermethylated in males relative to females before imposed weaning (BW) become hypomethylated in males and hypermethylated in females afterward (AW), resulting in near disappearance of the difference in methylation between the two sexes just after imposed weaning (AW; Figure 2e). The heat map in Figure 2f represents the changes in levels of DNA methylation in a group of genes that are differentially methylated between the sexes before (BW) and after imposed weaning (AW). This heat map reveals the intense difference in methylation of these genes before (BW) imposed weaning (intense and inverse red and blue signals between males and females), while after (AW) imposed weaning the DNA methylation levels of the genes are equalized (Figure 2f). The differences in methylation are reinstated later in the samples from 2 years of age, after the possibly stressful imposed weaning, reflecting a period of intense and inverse alterations in DNA methylation in both sexes. The data suggest different trajectories of evolution of DNA methylation patterns in males and females throughout the developmental stages leading into adolescence (Figure 2a).

Pathway analysis of the genes with DNA methylation differences between the sexes reveals interesting differences in functional pathways and upstream regulators of genes that maintain the same sex differences throughout development (see online only Supplementary Table S.2) as well as those that evolve during development (see online only Supplementary Table S.3 for early differences and Table S.4 for differences emerging at 2 years). The early DNA methylation differences affect stem cell and pluripotency pathways while the genes that become different later in development fall into pathways of cellular signaling (Figure 2g). During the period of weaning, genes that are differentially methylated between the sexes are targets of beta estradiol (see online only Supplementary Table S.5) pointing to a possible role of the female sex hormone during this period in development (Figure 2g).
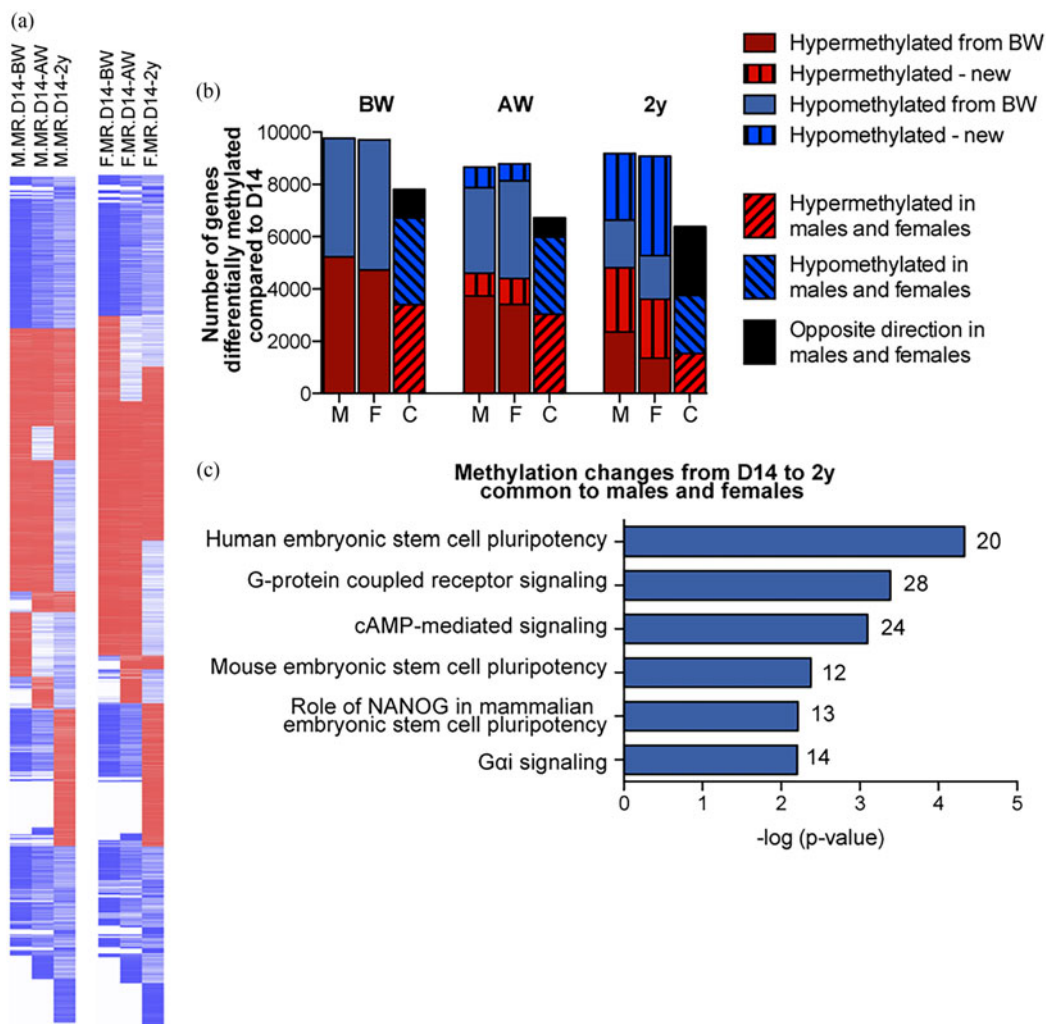
Figure 1. (Color online) Developmental changes of DNA methylation levels in CD3+ T cells of male and female mother-reared monkeys over the first 2 years of life. DNA methylation differences across gene promoters were calculated by averaging the methylation differences of significantly affected probes ($q < 0.2$) of each gene promoter region. (a) Clustering (Jaccard distance, average linkage) of genes whose promoters were differentially methylated in CD3+ T cells of mother-reared, control (MR) male (M) and female (F) monkeys at later stages of development, that is, before their imposed weaning (BW) at 6–7 months of age, after their weaning (AW) at 9–10 months of age, or after 2 years (2y) at 26–30 months of age as compared with their first weeks of life (D14: Day 14–30 samples). (b) Number of genes whose promoters were hypermethylated (indicated in red online only) or hypomethylated (indicated in blue online only) in CD3+ T cells of MR monkeys (M, F, common) in later developmental stages (BW, AW, and 2y) as compared to their first weeks of life (D14). The shading indicates whether the state of methylation was altered at the specific time point compared to BW (new) or remained the same since BW. (c) Top six canonical pathways associated with stable differentially methylated genes, that is, whose promoter methylation levels changed between the first weeks of life (D14) and 6 months of age, before the weaning period, and remained different till their second year of life (2y) in both male and female monkeys' CD3+ T cells.

Maternal deprivation early in life alters the normal evolution of DNA methylation profiles in a sex-dependent manner

We then determined whether the evolution of DNA methylation profiles is altered in monkeys subjected to different rearing conditions. Maternal deprivation affects the DNA methylation profiles at each of the developmental stages that were studied here and differences between surrogate-peer reared (SPR) and MR monkeys are observed in both sexes (see online only Supplementary Table S.6). The largest effects are observed at the early time point (Days 14–30) in

both males and females (Figure 3a, b). There is a large and highly significant overlap between genes that are altered by maternal deprivation at the early time period between males and females (Figure 3b). Nevertheless, there are clear sex differences in the profile of differentially methylated genes even at this early stage (Figure 3b). The differences in DNA methylation between MR and SPR monkeys is dynamic; new genes become differentially methylated during later stages of development and other genes revert to the level seen in MR animals (Figure 3b, e). The effect of maternal deprivation on the DNA methylation profile is reduced during developmental progression from D14 to the stage before
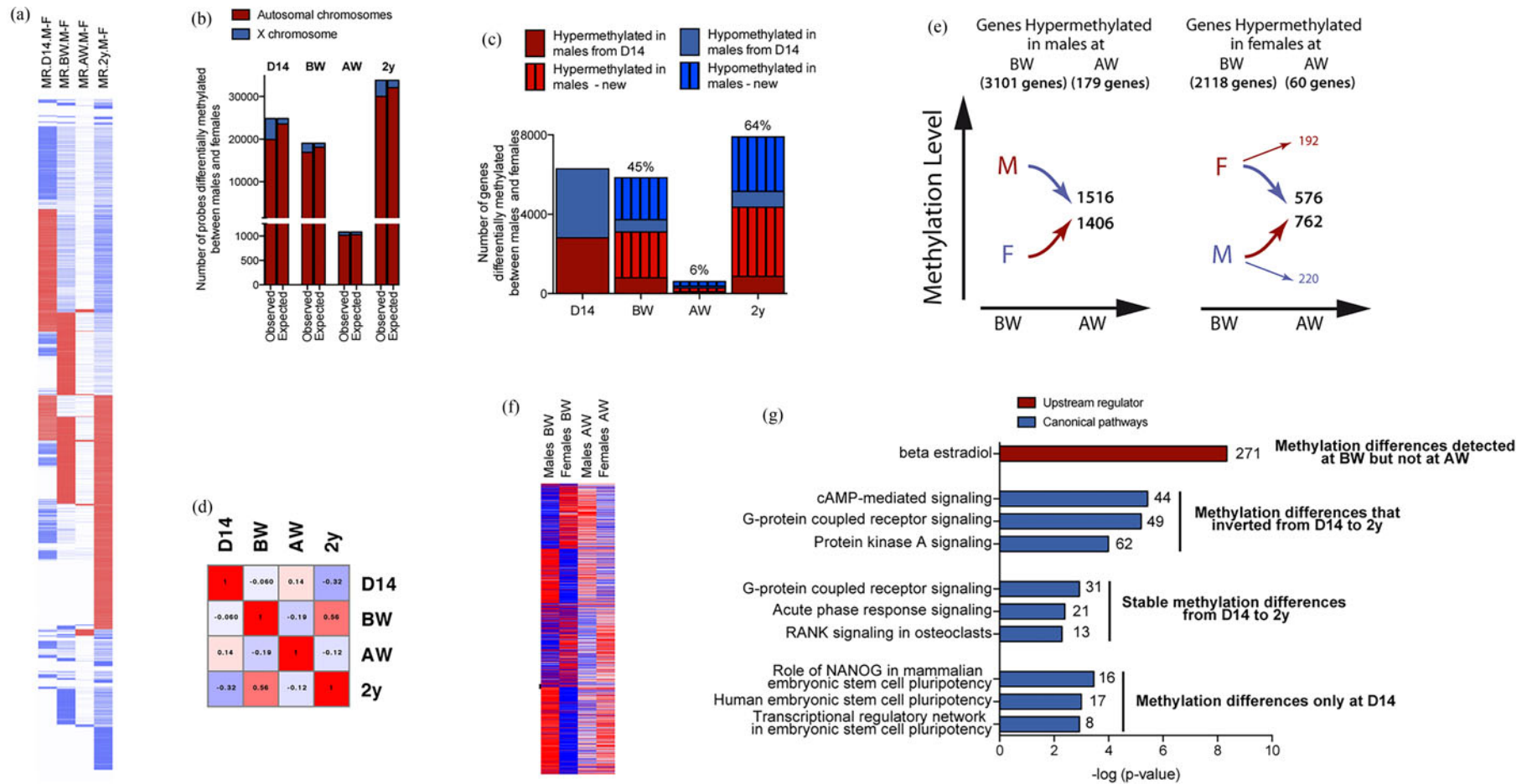
Figure 2. (Color online) DNA methylation differences between males and females in CD3+ T cells of mother-reared (MR) monkeys over the first 2 years of life. MR, Control monkeys, D14: Days 14–30 samples; BW, before weaning (6–7 months); AW, after weaning (9–10 months); 2y, after 2 years (26–30 months). The differences between males and females (M-F) are presented. (a) Clustering (row distance metric: Jaccard distance, average linkage) of genes whose promoters were differentially methylated ($q < 0.2$) between M and F MR monkeys' CD3+ T cells over their first 2 years of life. The colors in the online version correspond to the levels of differences (log 2). DNA methylation differences of gene promoters were calculated by averaging the methylation differences of significantly affected probes ($q < 0.2$) of each gene promoter region. (b) Number of probes differentially methylated ($q < 0.2$) between M and F MR monkeys' CD3+ T cells. The observed and expected ($p < .05$) distributions of probes between autosomal and sex chromosomes are indicated. (c) Number of genes whose promoters were hyper- and hypomethylated between M and F monkeys' CD3+ T cells over their first 2 years of life. The percentages above the bars at each time point indicate the proportion of genes whose promoters were already differentially methylated during their first month of life. The shading indicates whether the state of methylation was altered at the time point (new) or remained the same since Days 14–30. (d) Pearson product-moment correlation coefficients between averaged promoter methylation differences ($q < 0.2$) detected between M and F monkeys' CD3+ T cells during the sampled developmental stages (D14, BW, AW, and 2y). Significant coefficients ($p < .05$) are in bold. (e) Number of genes whose promoters were differentially methylated between M and F monkeys' CD3+ T cells BW and that remained differentially methylated AW. The numbers next to the arrows indicate the numbers of genes whose promoters were differentially methylated ($q < 0.2$) BW but not AW in either M or F monkeys. Blue and red arrows correspond to decreases and increases in methylation AW compared to BW, respectively. (f) Clustering (row distance metric: Pearson correlation, average linkage) of genes whose promoters were differentially methylated ($q < 0.2$) between M and F monkeys' CD3+ T cells BW but not AW. The colors correspond to the average methylation levels of the probes, for each promoter. (g) Top upstream regulators associated with genes differentially methylated between M and F monkeys BW but not AW (first row). Top canonical pathways associated with genes differentially methylated between M and F monkeys during their first 2 years of life in the same or opposite directions at 2 years old compared to the first month. The numbers at the right of the bars indicate the numbers of differentially methylated genes that are associated with the pathways or are known to be regulated by the upstream regulator.

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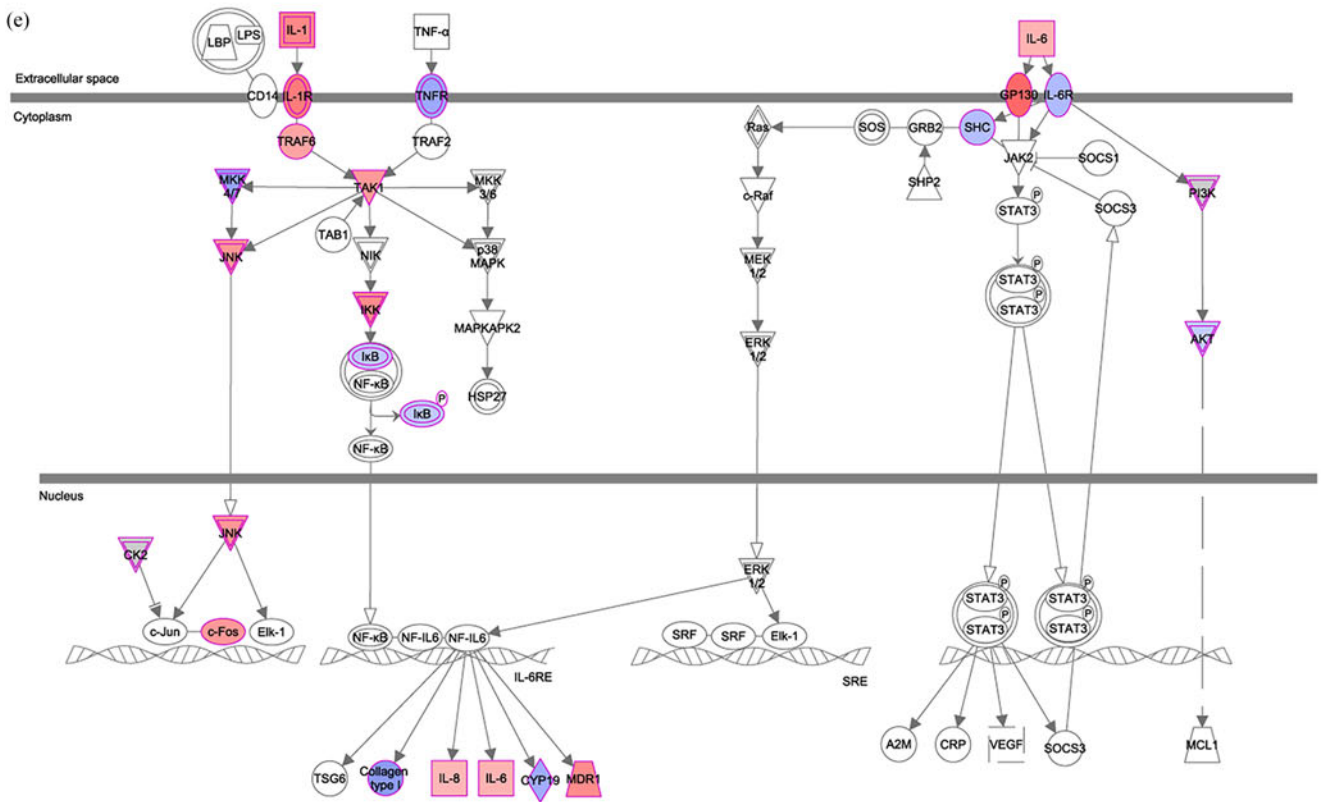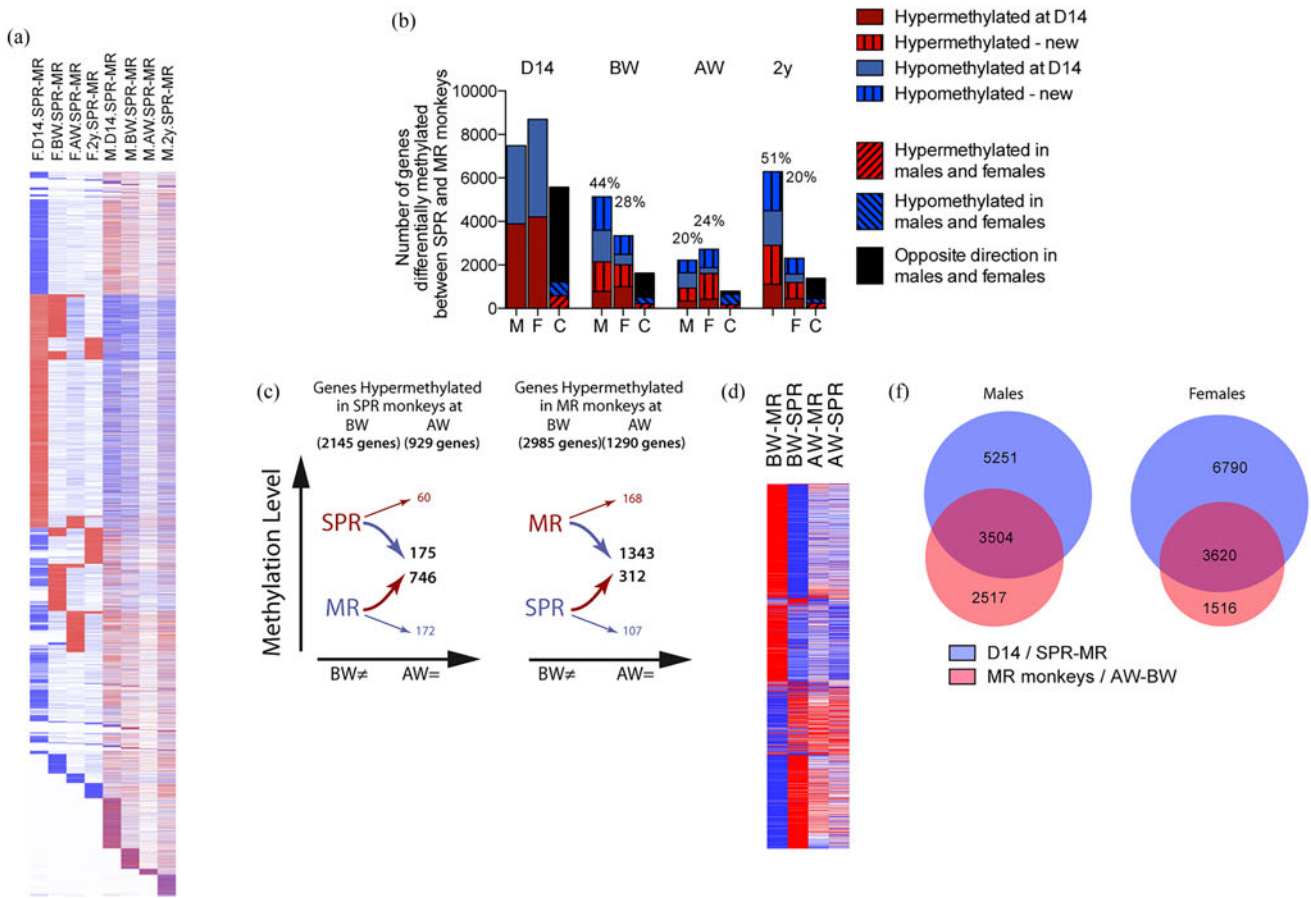
weaning with the most dramatic reversal in differences in DNA methylation occurring at the imposed weaning period (Figure 3b). Examination of the changes in the DNA methylation levels of differentially methylated genes between the male rearing groups before and after imposed weaning underscores the nature of changes in DNA methylation of these genes during imposed weaning (Figure 3b). Genes that are hypermethylated in SPR animals and hypomethylated in MR animals become less methylated in SPR animals after imposed weaning and more methylated in MR animals, resulting in equal intensity of DNA methylation in both groups after imposed weaning (Figure 3c, d). Differences in DNA methylation between the rearing conditions emerged again at 2 years mostly in males but less so in females (Figure 3a, b).

Examination of the canonical pathways that are differentially methylated before imposed weaning in male monkeys and are reversed by imposed weaning reveals major inflammatory pathways such as TNF-alpha, IL1, and IL6 (Figure 3e), as well as stress-related pathways including glucocorticoid receptor signaling and glucocorticoid biosynthesis (see online only Supplementary Table S.7).

Overlap between differentially methylated genes in surrogate-peer-reared monkeys at Day 14 and genes that are differentially methylated during imposed weaning at 9 months

That the difference in methylation between the two rearing groups is reversed at the time of weaning and that this is a consequence of changes in DNA methylation at the time of imposed weaning in naturally reared animals suggests that weaning at 7–8 months and maternal separation early after birth target an overlapping set of genes.

We determined therefore the overlap between genes that are differentially methylated during maternal separation after birth and those that are affected by imposed weaning in MR animals and found a highly significant overlap of 3,000 genes in both sexes (Figure 3f). There is an overlap of 59 upstream regulators of pathways significantly enriched with differentially methylated genes during imposed weaning and maternal separation upon birth at Day 14 (see online only Supplementary Table S.8). These include the stress hormone receptor NR3C1 and inflammatory and immune pathways such as IL1, IL4, NFATC2, STAT5, and TGFB1, as well as the sex hormone beta estradiol and DNA methylation enzymes DNMT1 and DNMT3A. This suggests that experimental maternal separation at birth targets similar genes in similar physiological pathways to those affected by the later stage separation from the mother during imposed weaning.

Examination of individual genes including important players of the stress response, such as *FKBP5* and *NR3C1* or the immune system, such as *CD3E* and *IL1R2*, showed dynamic changes with age, sex, and maternal rearing conditions (Figure 4a–c quantitative MeDIP analyses). It is important that the patterns of the differences in methylation levels between the sexes in the glutamate receptor *GRIA1* and interleukin receptor *IL1R2* genes were similar in the quantitative MeDIP and the array analyses (Figure 4b). For detailed validation of the array data, we selected differentially methylated genes from the maternal deprivation experiment, focusing on the first and last samples (D14 and 2y). The pyrosequencing analyses showed similar patterns to the MeDIP analyses (Figure 4c, d).

Discussion

A growing body of data suggests that early life experiences can trigger changes in DNA methylation that might be mediating long-term phenotypic effects (Szyf, 2011). The first evidence for such a process came from studies of maternal care in rats. These studies revealed epigenetic programming of the glucocorticoid receptor gene promoter (*nr3c1*) in response to maternal care in early life that serves as a lifelong stable genomic memory (Weaver et al., 2004). We addressed here the questions of whether such a simple mechanism of genomic memory represents a general rule for the changes in epigenetic programming occurring in response to experience during early life, and whether sex-specific effects emerged in these patterns.

Figure 3. (Color online) DNA methylation differences between mother-reared (MR) and surrogate-peer reared (SPR) monkeys' CD3+ T cells over the first 2 years of life. (a) Clustering (row distance metric: Jaccard distance, average linkage) of genes whose promoters were differentially methylated between the SPR and MR monkeys' CD3+ T cells. Female (F) and male (M) monkeys were analyzed separately at all sampled developmental stages (D14, first month; BW, before weaning; AW, after weaning; 2y, after 2 years). DNA methylation differences of gene promoters were calculated by averaging the methylation differences of significantly affected probes ($q < 0.2$) of each gene promoter region. (b) Number of genes whose promoters were hyper- and hypomethylated between SPR and MR monkeys' CD3+ T cells over their first 2 years of life (M, F, and common). The percentages above the bars at each time point indicate the proportion of genes whose promoters were already differentially methylated during their first month of life. The shading indicates whether the state of methylation was altered at the time point (new) or remained the same since Day 14. (c) Number of genes whose promoters were differentially methylated between MR and SPR M monkeys BW and that remained differentially methylated AW. The numbers next to the arrows indicate the numbers of genes whose promoters were differentially methylated ($q < 0.2$) BW and AW in either M or F monkeys. Blue and red arrows in the online version correspond to decreases and increases in methylation AW compared to BW, respectively. (d) Clustering (row distance metric: Pearson correlation, average linkage) of genes whose promoters were differentially methylated ($q < 0.2$) between MR and SPR male monkeys' CD3+ T cells BW but not AW. The colors online correspond to the average methylation levels of probes differentially methylated BW, for each promoter. (e) An example of canonical pathways associated with genes whose promoters were differentially methylated ($q < 0.2$) between MR and SPR M monkeys BW but not AW. The red and blue colors online indicate hyper- and hypomethylation in SPR monkeys compared to MR monkeys, respectively. (f) Overlaps between genes whose promoters were differentially methylated between SPR and MR monkeys during their first month of life (D14/SPR-MR) or between before and after the imposed weaning periods within MR monkeys (MR/AW-BW).
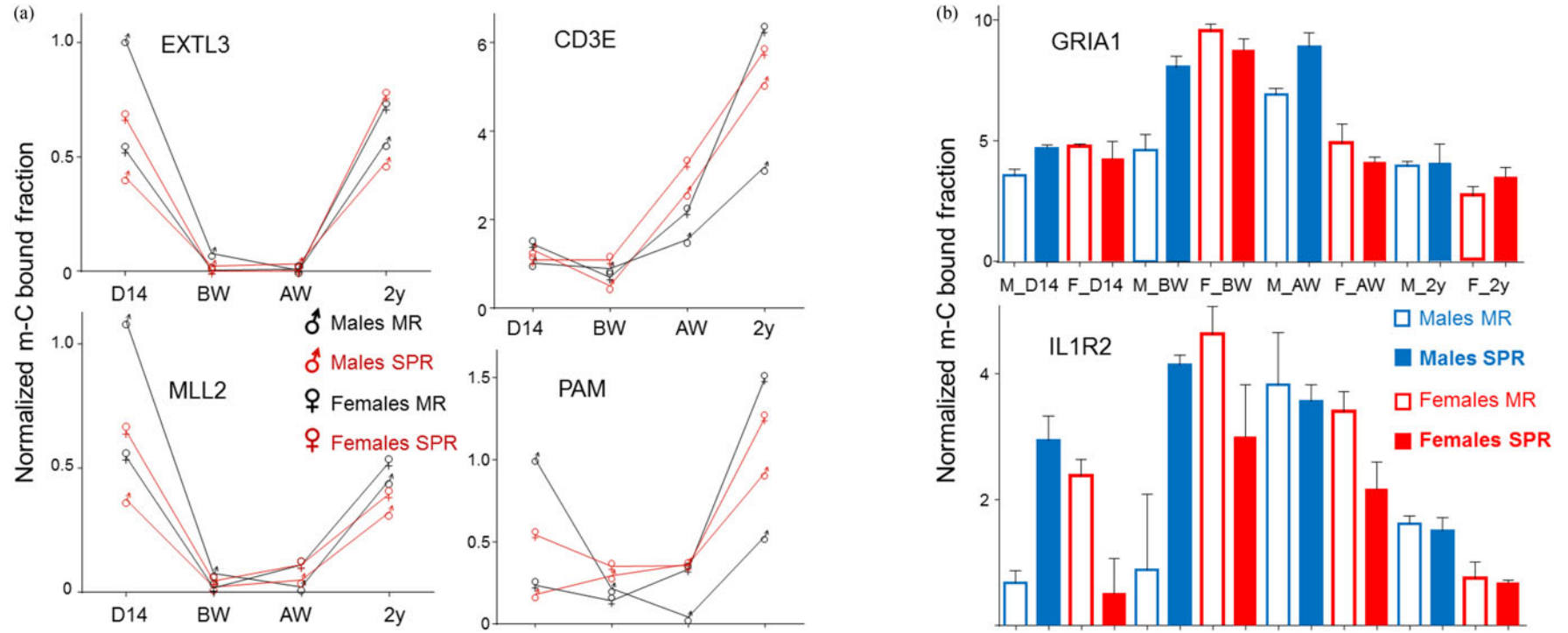
Figure 4. (Color online) Validation of DNA methylation differences. (a,b) Relative DNA methylation enrichment (normalized m-C bound fraction) of the whole genome amplified pooled samples used for the methylated DNA immunoprecipitation (MeDIP) arrays by quantitative MeDIP analysis (mean±SEM), **$p < .005$, *$p < .05$, and #$p < .1$ at Student t test. (c,d) DNA methylation levels of mother-reared and surrogate-peer reared groups from the first and last sampled developmental stages (Day 14 and 2 years) measured by quantitative MeDIP (normalized m-C bound fraction) and by pyrosequencing (mean methylation per rearing group per CG-site with the SEM), **$p < .005$, *$p < .05$, and #$p < .1$ at Student t test. In the online version, blue indicates male groups, red indicates female groups, and shading indicates surrogate-peer reared groups.
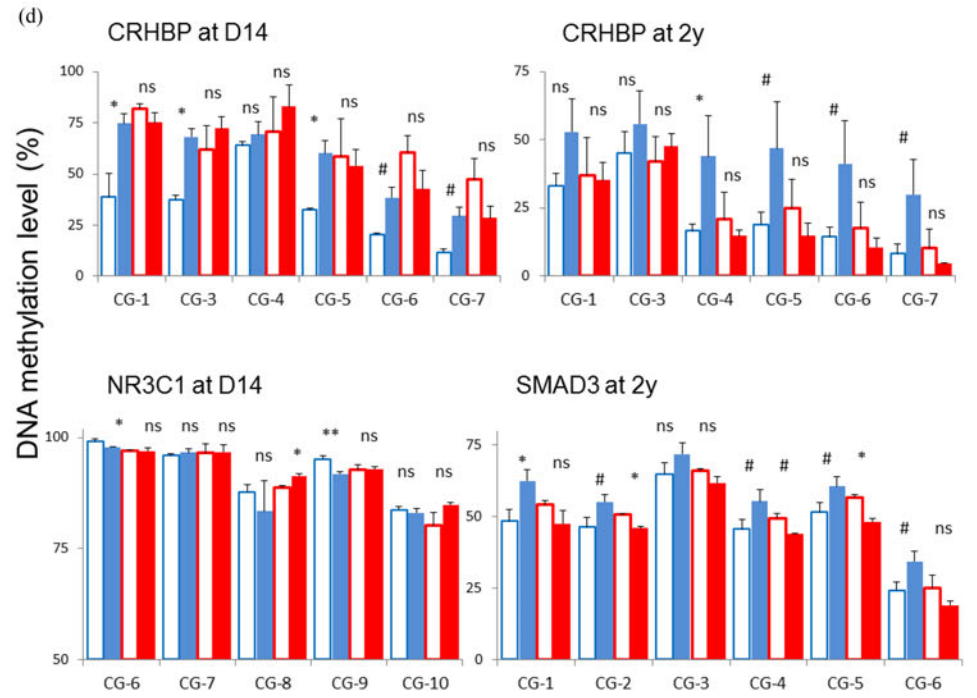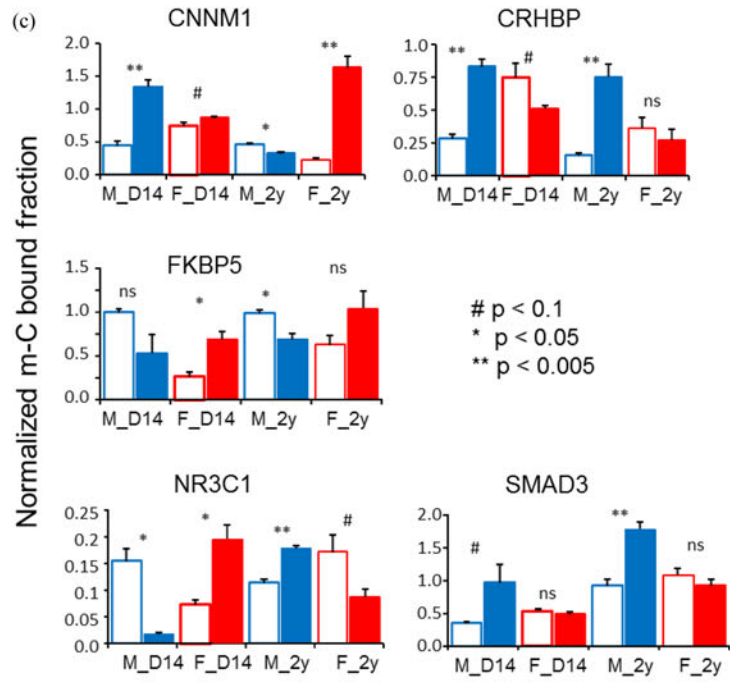
Figure 4. (*cont.*)

Previous studies in humans (Cao-Lei et al., 2014; Suderman et al., 2012) and nonhuman primates (Provencal et al., 2012) associated differences in DNA methylation with differences in early life experience in adults, but these studies did not address the developmental trajectories of these differences. It is possible that these changes emerged later in life in response to late life experiences. However, these changes in DNA methylation detected in adults may have evolved dynamically during postnatal development in response to the original early experience. Because the normal evolution of DNA methylation profiles during postnatal development was unknown, it was impossible to determine how the differences in DNA methylation detected in adulthood relate to early life proximal response of DNA methylation to experience. In addition, the effect of sex on normal development of DNA methylation in peripheral tissues such as the immune system was unknown.

To address these outstanding critical questions, we performed a longitudinal developmental study of DNA methylation profiles in T cells in rhesus monkeys from both sexes who were either reared with their mothers in social groups or separated from their mothers after birth and reared by human caregivers with peer socialization. Our study revealed that, first, DNA methylation profiles continue to dynamically evolve postnatally through the juvenile and up to the adolescent years. As development progresses, new genes become differentially methylated while others revert to the base line state of methylation (Days 14–30). Second, the evolution of DNA methylation pattern during development is different between sexes. The state of methylation of different genes is altered in hundreds of cases in reverse directions during development in males and females, and different genes are affected in males and females at the different time points. Third, early life separation from the mother alters the course of natural dynamic evolution of the DNA methylation pattern during development. The differences in DNA methylation triggered by early exposure continue to evolve as the animals mature and are different during adolescence compared to the changes seen at earlier stages of development. This implies that early life experience is registered as a dynamic memory in the DNA methylation profiles rather than a stable alteration and that changes in DNA methylation that appear only later in life were triggered by a dynamic sequence of changes elicited by an experience that occurred years earlier. This model of dynamic alteration of normal developmental trajectories of DNA methylation by early life experience could explain how the phenotypic impact of early life experience is frequently manifest only in later developmental stages or in adulthood (Sinclair, Lea, Rees, & Young, 2007).

Previous studies focused on changes in DNA methylation that appear early and are lifelong stable. We observe such genes in this study as well. Although the overall picture of experience-related DNA methylation alteration is dynamic through development, a small subset of genes ($\sim$45) shows consistent differences in methylation during development that are sex independent (see online only Supplementary Table S.9). These genes are involved in cellular signaling and might represent alterations in regulatory control in the SPR animals.

The differences in the dynamic evolution of the DNA methylation profiles between sexes is not limited to bona fide sex-related genes or the X chromosome. They appear in autosomal genes as well and affect basic immune and inflammation-related pathways. Thus, sex differences affect the epigenetic programming of apparently "sex neutral" functions such as inflammation and immunity, and these are evolving differently through postnatal development.

Our data suggest that dramatic changes in DNA methylation occur around an imposed weaning period in socially housed, MR primates. During this period changes in DNA methylation in reverse directions in males and females result in equalization of DNA methylation profiles between the sexes. Sex differences emerge again during adolescence.

These data suggest that gene pathways exist that are sensitive to maternal separation even later in development and may be destined to become differentially methylated. These alterations in epigenetic programming are perhaps behind the physiological and behavioral changes associated with imposed weaning (Dettmer et al., 2012). We show here an overlap between genes that are altered with maternal separation during imposed weaning later in development, and those that are precociously activated by early life maternal separation. Both later and neonatal maternal separation target changes in DNA methylation in overlapping genes. Nevertheless, the precocious interference with the typical developmental evolution of DNA methylation profiles leaves its mark in SPR adolescent primates and might in part explain possible pathologies that emerge later in life.

It is notable that although both sexes exhibited dynamic alterations to DNA methylation in response to maternal separation early at birth, males exhibited larger differences than females in the pattern of methylation during adolescence. This points to the possibility that differences in epigenetic responses might underlie the noted sex differences in phenotypic responses to early life events (Chaloner & Greenwood-Van Meerveld, 2013; Davis & Pfaff, 2014; Grassi-Oliveira, Honeycutt, Holland, Ganguly, & Brenhouse, 2016; Leussis, Freund, Brenhouse, Thompson, & Andersen, 2012).

Early life maternal separation is clearly a behavioral adversity; nevertheless, it leaves a broad and dynamic impact on DNA methylation in the immune system. These results extend an expanding body of data that links behavioral interventions and epigenetic alterations in peripheral tissues and particularly the immune system (Szyf, 2014). Collectively, the findings presented here have important practical and mechanistic implications for the field of behavioral epigenetics.

Supplementary Material

To view the supplementary material for this article, please visit http://dx.doi.org/10.1017/S0954579416000833.

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