

ORIGINAL ARTICLE

MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD

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Early life stress (ELS) is associated with increased vulnerability for diseases in later life, including psychiatric disorders. Animal models and human studies suggest that this effect is mediated by epigenetic mechanisms. In humans, epigenetic studies to investigate the influence of ELS on psychiatric phenotypes are limited by the inaccessibility of living brain tissue. Due to the tissue-specific nature of epigenetic signatures, it is impossible to determine whether ELS induced epigenetic changes in accessible peripheral cells, for example, blood lymphocytes, reflect epigenetic changes in the brain. To overcome these limitations, we applied a cross-species approach involving: (i) the analysis of CD34+ cells from human cord blood; (ii) the examination of blood-derived CD3+ T cells of newborn and adolescent nonhuman primates (*Macaca mulatta*); and (iii) the investigation of the prefrontal cortex of adult rats. Several regions in *MORC1* (MORC family CW-type zinc finger 1; previously known as: microrchidia (mouse) homolog) were differentially methylated in response to ELS in CD34+ cells and CD3+ T cells derived from the blood of human and monkey neonates, as well as in CD3+ T cells derived from the blood of adolescent monkeys and in the prefrontal cortex of adult rats. *MORC1* is thus the first identified epigenetic marker of ELS to be present in blood cell progenitors at birth and in the brain in adulthood. Interestingly, a gene-set-based analysis of data from a genome-wide association study of major depressive disorder (MDD) revealed an association of *MORC1* with MDD.

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INTRODUCTION

Early life stress (ELS) is associated with increased vulnerability for diseases in later life, including psychiatric disorders.^{1–8} Previous studies suggest that the effect of ELS on lifelong phenotypes is mediated by epigenetic mechanisms.^{9–11} Weaver *et al.*,¹² reported lifelong epigenetic modifications of the glucocorticoid receptor gene (*NR3C1*) in the rat hippocampus in response to stress induced by low levels of maternal care. More recent studies in rodents have reported ELS effects on DNA methylation for additional genes, including *AVP*, *BDNF*, *NR4A1*, *SLC6A4*, *HSD11B2*, *RELN*, *Esr1* and *CRH*.^{13–21} Similarly, in humans ELS has been shown to be associated with altered methylation of *NR3C1*, *BDNF* and *SLC6A4*.^{22–31} Recently, Klengel *et al.*³² reported that demethylation

of a glucocorticoid response element in the stress response regulator *FKBP5* predicted risk for posttraumatic stress disorder, and that this association was dependent upon risk allele and childhood trauma status. Although the above studies focused on candidate genes, two recent studies investigating ELS effects on an epigenome-wide level identified a variety of epigenetic alterations due to ELS.^{33,34}

Studies of ELS in humans are limited. First, since brains of living humans are not accessible for epigenetic studies, it is impossible to ascertain if alterations in DNA methylation of peripheral cells after exposure to ELS reflect brain DNA methylation changes triggered by ELS. Second, excluding underlying causes of changes in DNA methylation other than ELS is problematic, as genetic

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background and/or predisposing environmental factors cannot be randomized and controlled in human studies. And third, to address the issue of a temporal relationship between ELS, DNA methylation changes and adult phenotypes, a longitudinal investigation of a human cohort from birth until adulthood would be required.

To partially address these limitations, we investigated the effects of ELS on the methylome using a convergent approach. This involved: (i) a case-control analysis of human CD34+ cells from cord blood; (ii) the examination of CD3+ T cells from the peripheral blood of nonhuman primates (*Macaca mulatta*) at ages 14–30 days and 2 years; and (iii) the analysis of the prefrontal cortex (PFC) of adult rats.

To limit the influence of later environmental factors in human studies that might affect DNA methylation independently of ELS, an investigation as early in life as possible must be performed. We analyzed cord blood, as this is the earliest accessible biomaterial in humans although we recognize that the DNA methylation pattern might be impacted intrauterine by multiple exposures. We focused on CD34+ cells, progenitors of white blood cells, as white blood cells have an important role in the immune response of an individual, and they are therefore likely to bear environmental-exposure-related DNA methylation signatures.³⁵ Any DNA methylation state that is triggered by prenatal stress and persists later in life in white blood cells must have originated in progenitor CD34+ cells as they contribute throughout life to the renewing pool of mature peripheral white blood cells. However, additional cell-specific DNA methylation changes, which are not present in CD34+ cells at birth, are likely to occur during cell differentiation.

We identified five single-copy genes, whose promoter DNA methylation state of individual transcripts is affected the same way (hypo- or hypermethylation, respectively) after exposure to ELS in all the three species, at all the investigated time points and, in the peripheral tissues, as well as in the rodent brain. A gene-set-based analysis of available genome-wide association study (GWAS) data on major depressive disorder (MDD)³⁶ revealed an association between one of those genes, *MORC1* (MORC family CW-type zinc finger 1) and human depression.

MATERIALS AND METHODS

For detailed methodological descriptions, see Supplementary Information.

Human cohort

Data were obtained from a cohort of mothers and their infants ($n = 180$) recruited during the third trimester of pregnancy in the Rhine-Neckar Region of Germany. For inclusion and exclusion criteria see Supplementary Information. The study protocol was approved by the Ethics Committee of the Medical Faculty Mannheim of the University of Heidelberg. The study was conducted in accordance with the Declaration of Helsinki. All mothers provided written informed consent before participation.

A structured interview and questionnaires were used for risk factor assessment (Supplementary Table S1a). A composite score was calculated to take three different dimensions of stress into account: (a) maternal psychopathology (primarily depressive and anxiety symptoms); (b) perceived stress; and (c) socioeconomic and psychosocial stress (Supplementary Table S1c). Stressful prenatal adverse conditions were also considered to define 10 infants with extremely high and 10 infants with extremely low levels of prenatal ELS, respectively. The sociodemographic and medical characteristics of the extreme groups are shown in Supplementary Table S1b. For the comparison of the extreme groups, two-tailed *t*-tests for independent samples were used. All results are expressed as means \pm standard deviation or as a percentage, as appropriate. The epigenome-wide data sets of two infants in the low ELS group did not pass our quality control filters, therefore, the group size decreased to $n = 8$.

Animals

Rhesus monkeys were reared as previously described.³⁵ Venous blood samples were obtained from 14–30 days old and 2-year-old monkeys.

Pregnant rats were randomly assigned to control (Ctrl; $n = 7$) or prenatal stressed (PS; $n = 9$) conditions. The stress paradigm was carried out as previously described.³⁷ Male offspring PFCs were dissected on postnatal day 62.

In the case of rhesus macaques, protocols for the use of experimental animals were approved by the Institutional Animal Care and Use Committee of the NICHD. Rat handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92). All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Separation of CD34+ cells from human cord blood

Cord blood was collected immediately after birth and drawn into ethylenediaminetetraacetic acid (EDTA)-coated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated through centrifugation with Ficoll-Paque (GE Healthcare, Munich, Germany), and CD34+ cells were extracted from PBMCs using the Dynal CD34 Progenitor Cell Selection System (Life Technologies, Darmstadt, Germany).

Separation of CD3+ T cells from monkey peripheral blood

This was performed as previously described.³⁵ In brief, peripheral blood was drawn into EDTA-coated tubes. PBMCs were isolated through centrifugation with Ficoll-Paque (GE Healthcare, Burnaby, BC, Canada), and T cells were isolated from the PBMCs using CD3+ Dynabeads (Life Technologies, Burlington, ON, Canada).

Extraction of DNA

Genomic DNA was extracted using Qiagen (Hilden, Germany) or Promega (Madison, WI, USA) systems, sheared by sonication and quantified using the Qubit system (Life Technologies, Burlington, ON, Canada).

Analysis of genome-wide promoter DNA methylation

The procedure used for MeDIP analysis was adapted from previously published protocols.^{35,38} Briefly, 2 μ g of DNA were sonicated, and methylated DNA was immunoprecipitated using anti-5-methyl-cytosine (Eurogentec, Fremont, CA, USA). The DNA-antibody complex was immunoprecipitated with protein G, and the methylated DNA was resuspended in digestion buffer (50 mM TRIS-HCl pH8; 10 mM EDTA; 0.5% SDS) and treated with proteinase K overnight at 55 °C. The input and bound fraction were purified, amplified using the Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA), and labeled for microarray hybridization with Cy3-dUTP and Cy5-dUTP, respectively, using the CGH Enzymatic Labeling Kit (Agilent Technologies, Mississauga, ON, Canada) in accordance with the manufacturer's instructions. Custom designed tiling arrays were used (Agilent Technologies). All steps of the hybridization, washing, scanning and feature extraction procedures were performed in accordance with the Agilent Technologies protocol for chip-on-chip analysis. Extracted microarray intensities were processed and analyzed using the R software environment for statistical computing (<http://www.r-project.org/>).

Validation

Gene-specific validation of MeDIP data was performed applying quantitative-real time PCR (QPCR) using the $2^{-\Delta\Delta C_t}$ method. Data are expressed as group means \pm s.e.m. The Graphpad 5 software was used to perform one-tailed Mann-Whitney *U*-tests.

RNA extraction from human cord blood

Cord blood for RNA extraction was collected immediately after birth and drawn into PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland). RNA was extracted using the PAXgene Blood RNA Kit (Qiagen). The quality and quantity of RNA samples were analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). For one of the infants from the high ELS group, no RNA was available for analysis.

Analysis of gene expression in human cord blood

Reverse transcription was performed using the high-capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany). Gene expression levels were analyzed using QPCR and the $2^{-\Delta\Delta Ct}$ method. Data are expressed as group means \pm s.e.m. Statistical significance was tested using one-tailed Mann–Whitney *U*-tests.

Pyrosequencing

The promoter region of *MORC1* (NC_000003.11, assembly: CRCh37/hg19, position: 108,838,104–108,838,644) was analyzed by pyrosequencing. In brief, three fragments of bisulfite-treated DNA (EpiTect Bisulfite Kit, Qiagen) were amplified by PCR (HotStar Taq DNA Polymerase, Qiagen; primer information see Supplementary Table S2) using an unmodified forward primer and a biotin-labeled reverse primer (Eurofins, Ebersberg, Germany). Pyrosequencing was performed using a PyroMark Q24 Advanced system (Qiagen; primer information see Supplementary Table S2) in accordance with the manufacturer's protocol. Methylated and unmethylated EpiTect control DNA samples (Qiagen) were used as controls for bisulfite conversion, amplification and pyrosequencing. The percentage of methylation at each CpG site was quantified using the PyroMark Q24 Advanced software version 3.0.0 (Qiagen) Sequencing was performed in triplicate. Quality control filtering and statistical analyses of the pyrosequencing results were conducted using R Version 2.15.3 (<http://www.r-project.org>). Measurements marked as unreliable by the Pyromark software were removed from the data set. Triplicate measurements were averaged after the removal of outliers (values deviating more than 3%). A Mann–Whitney *U*-test was used to compare the mean percentage of methylation of CpG sites for the ELS versus control groups. Data are presented as the mean \pm s.e.m.

Genetic analysis

Genotypes were investigated using GWAS data of a previous study of MDD.³⁶ dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>) was searched for SNPs (single-nucleotide polymorphisms) across the genes. The set-based test, as implemented in PLINK (v1.0.7)³⁹ was performed (with default options and 10^5 permutations), to test for association between MDD and the whole set of genetic variants in the data set.

RESULTS

Effects of ELS on genome-wide promoter methylation in human newborns

To shortlist candidate genes that: (i) are affected by ELS in humans, rats and monkeys; (ii) persist into adulthood; and (iii) are differentially methylated in both peripheral tissue and the brain, a series of MeDIP genome-wide methylation analyses were performed. For the human sample, CD34+ hematopoietic stem cells derived from the umbilical cord blood of extreme ELS groups were analyzed. A total of 3405 distinct genes were significantly associated with prenatal stress. Of these genes, 1786 were hypomethylated and 1750 were hypermethylated after exposure to ELS. For 131 genes, a mixed methylation pattern was observed. Supplementary Table S3a provides a list of genes associated with the 25 most significant probes. Interestingly, this list includes *B3GAT2* for which an association with schizophrenia has been previously reported.⁴⁰

Effects of ELS on genome-wide promoter methylation in peripheral tissues of nonhuman primates

Persistent changes in gene methylation secondary to ELS exposure that can be identified in venous blood cells were of particular interest, as these genes are of potentially high value in follow-up studies in humans. Venous blood of human infants cannot be obtained for ethical reasons. Therefore, the ELS signature of CD3+ T cells derived from venous blood of newborn (14–30 days old) and adolescent (2 years old) rhesus monkeys exposed to different rearing conditions (maternally reared versus surrogate-peer reared) have been analyzed. CD3+ T cells have

been chosen, as CD34+ stem cells, which are progenitors of CD3+ T cells, are not sufficiently abundant in venous blood.

In the CD3+ T cells of 14–30-day-old monkeys, a total of 4924 distinct genes were significantly associated with postnatal stress. Of these genes, 2803 were hypomethylated and 2424 were hypermethylated in surrogate-peer reared monkeys and 303 genes displayed a mixed methylation pattern. Supplementary Table S3b provides a list of genes associated with the 25 most significant probes.

In the CD3+ T cells of adolescent monkeys (2 years old), a total of 2547 distinct genes associated with postnatal stress have been identified. Of these genes, 1744 were hypomethylated and 873 were hypermethylated in surrogate-peer reared monkeys and 70 genes displayed a mixed methylation pattern. Supplementary Table S3c provides a list of genes associated with the 25 most significant probes.

A total of 1180 genes were differentially methylated in both the newborn and adolescent monkeys ($P < 2.6E - 10$, hypergeometric test), supporting the hypothesis that ELS is associated with a pervasive signature in the methylome of CD3+ T cells, which arise early after exposure, and that a number of methylation changes persist later in life.

A comparison of genes differentially methylated in human CD34+ with genes differentially methylated in peripheral CD3+ T cells of newborn and adolescent monkeys revealed an overlap of 176 genes (Supplementary Table S4). Gene set analysis of these 176 genes showed enrichments for biological functions such as cancer ($P = 4.45E - 6$) or gene expression ($P = 7.44E - 5$; data not shown) suggesting that the overlap may not be random, although it is not significant.

Identification of conserved genes responding to ELS in peripheral tissues and in the brain

To identify which of these overlapping genes are differentially methylated in the adult brain, the human and monkey data were compared with genome-wide methylation data derived from PFC of adult rats. A total of 3385 distinct genes were significantly associated with prenatal stress. Of these genes, 1554 were hypomethylated, 1973 were hypermethylated after exposure to ELS and 142 genes presented a mixed methylation pattern. Supplementary Table S3d provides a list of genes associated with the 25 most significant probes.

By comparing the data derived from human and monkey peripheral tissue with the rodent PFC data, we identified 30 genes (overlap not significant) whose methylation status was associated with ELS in all the tissues and species analyzed (Supplementary Table S5). Interestingly, this list includes *CACNA1C* one of the best-supported and replicated risk genes for affective disorders.^{41–44} By restricting the analyses to promoter regions (–2000 to +500 from the transcription start site) of individual transcripts, we identified seven gene promoters whose DNA methylation status is affected the same way (hypo- or hypermethylation, respectively) in all species and tissues. *U6*, *PDE4DIP*, *ADARB2* and *MORC1* were hypomethylated in the ELS groups. *7SK*, *PRMT5* and *CSRNP3* were hypermethylated in the ELS groups.

Association with depression

MDD is one of the well-established ELS-associated phenotypes.^{1–4} We therefore took advantage of a previous GWAS study on MDD³⁶ and performed a gene-based case–control analysis to test for an association between genetic variants in those overlapping genes and MDD. We excluded *U6* and *7SK* from our analysis, as multiple copies of those genes exist throughout the genome. In addition, we had to exclude *PRMT5* and *PDE4DIP* as no genetic variants in *PDE4DIP* and only one genetic variant in *PRMT5* were represented in the quality-controlled GWAS data set. Of the remaining three genes, *MORC1* and *CSRNP3* showed a nominally significant

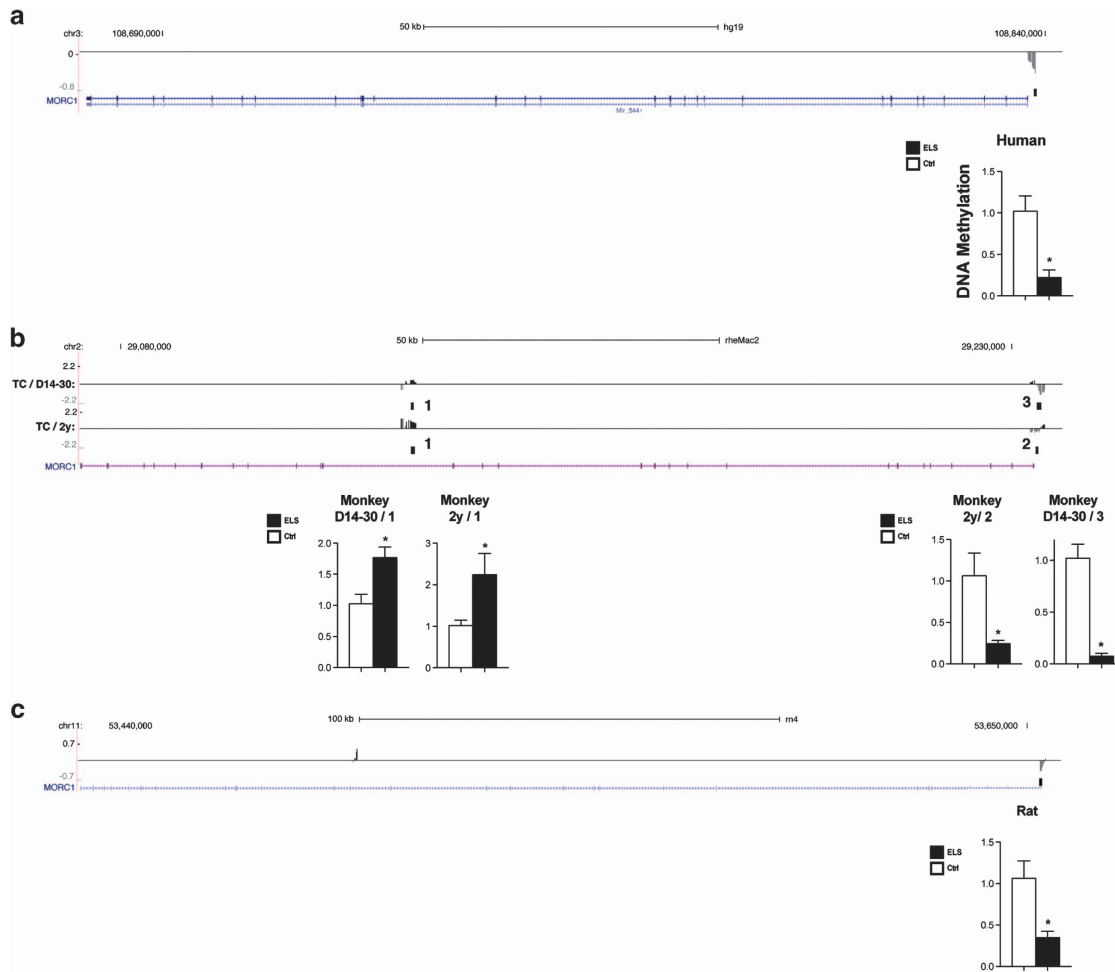


Figure 1. Expanded views from the UCSC genome browser of the *MORC1* gene are depicted. **(a)** Human CD34+ cord blood. **(b)** 14–30-day-old monkey CD3+ T cells (TC/D14–30), 2-year-old monkey CD3+ T cells (TC/2y). Numbers 1, 2, 3 indicate the locations of DNA amplification for QPCR validations. **(c)** Rat PFC. For each graph, average methylation probe fold differences (Log₂) between control and ELS groups, as well as regions of significant differential methylation are shown. The last track shows the *MORC1* gene, as taken from the NCBI reference sequences collection (RefSeq). QPCR analysis of DNA methylation differences in the *MORC1* gene between ELS and control groups are displayed. Relative bound fraction concentrations are shown. Error bars represent s.e.m. The symbol ‘*’ denotes *P*-values < 0.05, as calculated with the Mann–Whitney *U*-test. Ctrl, control group; ELS, early life stress; *MORC1*, *MORC* family CW-type zinc finger 1; QPCR, quantitative PCR.

association with MDD ($P=0.00483$ and 0.03139 , respectively). Only the association of *MORC1* with MDD withstood Bonferroni correction for the number of genes tested ($P=0.01449$), thus providing evidence that *MORC1* is involved in MDD, a stress-associated disorder. QPCR was used to validate *MORC1* methylation changes observed in human CD34+ cells, monkey CD3+ T cells and rat PFC (Figure 1).

In the human cohort, we attempted to validate the MeDIP results using a pyrosequencing approach. Pyrosequencing revealed very high methylation levels and the significant difference between the ELS and controls was not replicated (Figure 2).

MORC1 gene expression

To investigate whether the observed alterations in *MORC1* methylation levels secondary to ELS resulted in differential gene expression, *MORC1* expression levels were analyzed in cord blood samples derived from the human extreme groups using a QPCR

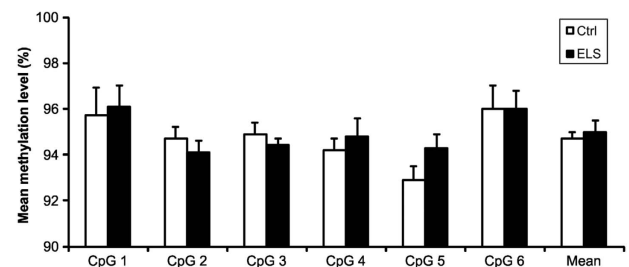


Figure 2. The bar graph shows the mean methylation levels in the ELS and control group for each of the six CpG sites in *MORC1*, as determined by pyrosequencing. In addition, the average methylation level of all sites is displayed. Error bars represent s.e.m. No significant difference between the ELS and Ctrl group was detected. Statistical significance was calculated using the Mann–Whitney *U*-test. Ctrl, control group; ELS, early life stress; *MORC1*, *MORC* family CW-type zinc finger 1.

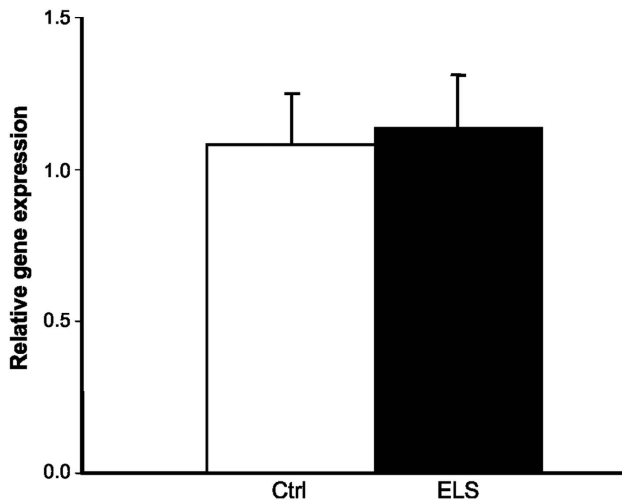


Figure 3. Relative expression of *MORC1* in human cord blood ($n=9$ ELS and $n=8$ Ctrl). Values are given as mean quantities normalized by β -actin. Error bars represent s.e.m. No significant difference between the ELS and Ctrl group was detected. Statistical significance was calculated using the Mann–Whitney *U*-test. Ctrl, control group; ELS, early life stress; *MORC1*, *MORC* family CW-type zinc finger 1.

approach. No differences in *MORC1* expression were detected between the high and low ELS groups (Figure 3).

DISCUSSION

The present study is the first to apply a cross-species and cross-tissue approach to the identification of ELS effects on the epigenome. As has been postulated before,⁴⁵ our data provide evidence for genome-wide and system-wide changes in DNA methylation in response to ELS. This information might be useful in predicting lifelong behavioral and physical phenotypes, especially as we could identify such changes very early in life. In this study, differential methylation of 30 genes was associated with ELS immediately after birth in human CD34+ cells, and additionally in peripheral CD3+ T cells of newborn and adolescent monkeys, as well as in the brain of adult rats. A gene-based case–control analysis to test for an association between genetic variants and MDD identified one of those genes—*MORC1*—as being additionally associated with MDD on a genetic level in a different cohort. Our results provide evidence for a link between the *MORC1* gene and depression. *MORC1* is an excellent candidate as an immediate ELS responsive gene as it is differentially methylated in the brain and peripheral cells at different ages and could be followed longitudinally in living humans.

Although previous studies reported associations between ELS and DNA methylation in the adult brain tissue or peripheral blood cells, critical questions remained unanswered. First, are ELS-associated DNA methylation changes a consequence of early life experiences, or a cause of the later psychiatric phenotypes resulting from ELS? To address this question, we examined changes in DNA immediately after exposure to ELS. Our data show that in both, monkeys and humans, DNA methylation differences emerged soon after ELS exposure supporting the hypothesis that DNA methylation changes follow ELS, and precede the appearance of the clinical phenotypes later in life.

In humans, disentangling DNA methylation caused by ELS from DNA methylation caused by other preexisting confounding factors such as genetic variation and other environmental factors remains impossible. We bypassed these limitations by applying a

convergent, translational approach comparing human data with data derived from animal models as they can be randomized to high and low ELS and their environment can be controlled.

A wide variety of prenatal stressors—including maternal depression during pregnancy, as well as a multitude of socioeconomic and psychosocial stressors—may result in epigenetic alterations and long-lasting effects on the health and behavior of the child. Accordingly, the present study applied a broad definition of prenatal stress in humans. However, all subjects in the high stress group were chronically exposed to multiple socioeconomic and psychosocial stressors as well as to high levels of subjectively perceived stress of the mother. The *Macaca mulatta* analyses focused on the effects of very early postnatal stress on the epigenome. This experimental condition was specific, standardized and adequately controlled. However, the comparison of a postnatal stress paradigm in the monkey to a gestational stress paradigm in humans could be considered a limitation of our study. Nonetheless, the maternal separation model for ELS applied in the nonhuman primates leads to neurobiological, physiological and behavioral consequences similar to those identified in humans after exposure to early adversity.^{46–49} Our study provides additional evidence that ELS causes evolutionary conserved differential methylation of responsive genes as has been postulated previously.⁵⁰

Although we are aware of sex-specific epigenetic effects, we decided to study the human offspring of the most stressed mothers, independent of sex of the infants. As the gender distribution did not differ significantly between the groups, we would consider this a minor issue. Maternal smoking and alcohol consumption during pregnancy have an impact on the global DNA methylation pattern of the infant.^{51–57} Both were significantly higher in the present high ELS group, and this is an obvious confounding factor. However, this effect can be excluded if overlapping differentially methylated genes are found in animal models of ELS, since these animals were exposed to neither cigarette smoke nor alcohol during gestation.

We reasoned that there exist robust and fundamental changes in DNA methylation, which will be conserved not just among species but also among tissues. The overlapping changes identified in both CD34+ and CD3+ cells are consistent with the hypothesis that changes in methylation in response to ELS appear early in progenitor cells which are then passed on to their different daughter lineages. Identification of genes that respond similarly to ELS in brain and blood cells is critical for studying behavioral epigenetics in humans and for potential diagnostics and therapeutic interventions. Future studies will be necessary to identify which specific cerebral cell subtypes are affected by ELS, as we analyzed PFC material without isolating specific cells.

ELS is a condition associated with several health and psychiatric conditions including depression in later life.^{1–4} Taking advantage of an available GWAS in MDD, we were able to demonstrate that genetic variants in one of those persistently methylated genes, *MORC1*, are significantly associated with MDD. This supports the hypothesis that *MORC1* is involved in at least one known consequence of ELS, the risk for depression in later life. The genome-wide finding for *MORC1* was confirmed in all the three organisms using QPCR, which is the standard method for validation of MeDIP-chip data. Unexpectedly, the additional attempt to replicate the differential methylation of *MORC1* using a pyrosequencing approach failed. One possible reason could be that the MeDIP-chip does not provide single base resolution, but instead suggests a region between 800 and 1200 bp harboring CpG sites of interest. The identification of differentially methylated CpGs in this area via pyrosequencing is hampered by the relative short (≤ 300 nucleotides) sequencing reads, which complicates selection of the target region for replication. It is therefore possible that we missed the differentially methylated CpGs. Furthermore, results obtained by MeDIP and bisulfite-based

sequencing are not necessarily comparable. For example, in a recent study by Jenke *et al.*,⁵⁸ methylation levels obtained by MeDIP differed substantially from methylation levels obtained by pyrosequencing. Furthermore, bisulfite conversion of DNA does not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine, whereas MeDIP using an anti-5-methylcytosine is selective for DNA methylation. We are therefore unable to exclude the possibility that the region identified by MeDIP as being differentially methylated after ELS is additionally differentially hydroxymethylated after ELS. This could furthermore explain the very high methylation levels obtained by pyrosequencing.

No significant differences in *MORC1* expression were detected in cord blood samples from the human extreme groups. Unfortunately, no RNA from CD34+ cells was available for our cohort. Hence gene expression analysis was performed in whole blood samples, whereas DNA methylation was measured in CD34+ cells. The cell-type-specific nature of DNA methylation patterns could therefore explain this unexpected finding. A recent study showed that hypermethylated promoters can be transiently activated through chromatin remodeling without promoter demethylation, thus contradicting the common hypothesis that correlates DNA hypermethylation with silencing of gene expression.⁵⁹ This finding suggests that promoter DNA methylation might be a programming factor providing an enduring memory for gene silencing rather than a marker of active gene expression. Further research is needed to ascertain if this phenomenon also applies to *MORC1* and to investigate the functional consequences of differential *MORC1* methylation in more detail.

The role of *MORC1* is largely unknown. In mammals, *MORC1* is mainly expressed in male germ cells. In the present study, *MORC1* expression levels in human cord blood were very low, and this may have hampered the detection of significant differences between the groups. Germ cell *MORC1* expression commences in early embryonic development and is important for the completion of prophase of meiosis I during spermatogenesis.^{60,61}

Loss of function mutation in this gene leads to male infertility in mice. For other members of the gene family, expression is not restricted to the testis, which suggests a more general biological function for the *MORC* gene family. Recent research suggests that *MORC1* itself has a more general biological role, as it codes for an evolutionary conserved nuclear protein, which may influence gene silencing and chromatin structure, possibly through the detection of epigenetic marks.^{62–64} The identification of *MORC1* as a gene whose promoter is differentially methylated after exposure to different forms of ELS in different organisms, different tissues and different time points also suggests that this gene has a more general regulatory role in response to stress. However, as only limited knowledge concerning the role of *MORC1* in epigenetic processes is available, the role of *MORC1* within the context of ELS remains a matter of speculation. Systematic investigation of this question is warranted.

In addition to the identification of *MORC1* as epigenetically modified after exposure to ELS and associated with MDD, differential methylation in the human sample was observed for *CACNA1C*, *ANK3* and *PCLO*, genes that were identified in previous GWAS and replication studies of affective disorders.^{41–43,65}

In conclusion, our novel systematic, genome-wide and cross-tissues-cross-species investigation of the effects of ELS on the epigenome identified *MORC1* as differentially methylated in all the three investigated organisms, in peripheral tissues as well as in the brain and at different time points in the life-span. Furthermore, an association was demonstrated between *MORC1* and MDD, an ELS-associated disorder. We therefore propose *MORC1* as a new candidate gene for stress-related disorders whose DNA methylation status: (i) reflects ELS; (ii) is amenable to longitudinal follow-up in peripheral cells; and (iii) may predict the emergence of ELS-

related disorders, such as depression, in later life. This has important research, diagnostic and therapeutic implications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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