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Exposure to different early-life stress experiences results in differentially altered DNA methylation in the brain and immune system

Clarissa Catale [a,1], Silvia Bussone [b,1], Luisa Lo Iacono [c,d], Maria Teresa Viscomi [e], Daniela Palacios [d], Alfonso Troisi [f], Valeria Carola [b,d,*]

[a] Department of Psychology, Ph.D. Program in "Behavioral Neuroscience", Sapienza University of Rome, Rome, Italy
[b] Department of Dynamic and Clinical Psychology, Sapienza University of Rome, Rome, Italy
[c] Department of Psychology, Sapienza University of Rome, Rome, Italy
[d] IRCCS Santa Lucia Foundation, Rome, Italy
[e] Department of Life Science and Public Health, Section of Histology and Embryology, University "Cattolica Del S. Cuore", Rome, Italy
[f] Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy

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ABSTRACT

The existence of a proportional relationship between the number of early-life stress (ELS) events experienced and the impoverishment of child mental health has been hypothesized. However, different types of ELS experiences may be associated with different neuro-psycho-biological impacts, due to differences in the intrinsic nature of the stress. DNA methylation is one of the molecular mechanisms that have been implicated in the "translation" of ELS exposure into neurobiological and behavioral abnormalities during adulthood. Here, we investigated whether different ELS experiences resulted in differential impacts on global DNA methylation levels in the brain and blood samples from mice and humans. ELS exposure in mice resulted in observable changes in adulthood, with exposure to social isolation inducing more dramatic alterations in global DNA methylation levels in several brain structures compared with exposure to a social threatening environment. Moreover, these two types of stress resulted in differential impacts on the epigenetic programming of different brain regions and cellular populations, namely microglia. In a pilot clinical study, blood global DNA methylation levels and exposure to childhood neglect or abuse were investigated in patients presenting with major depressive disorder or substance use disorder. A significant effect of the mental health diagnosis on global methylation levels was observed, but no effect of either childhood abuse or neglect was detected. These findings demonstrate that different types of ELS have differential impacts on epigenetic programming, through DNA methylation in specific brain regions, and that these differential impacts are associated with the different behavioral outcomes observed after ELS experiences.

1. Introduction

Child maltreatment (CM) is defined as any act of commission or omission by a caregiver that results in harm, the potential for harm, or the threat of harm to a child. The impact of CM is intense and harmful, with neurobiological and neuroendocrine consequences, and CM can affect all aspects of child development, including behavioral, emotional, social, physical, and cognitive aspects (Danese and Lewis, 2017; Bremne and Vermetten, 2001). CM has common and powerful associations with the occurrence of various types of psychiatric disorders and has become a strong predictor of mental illness (Teicher and Samson, 2013). Classically, CM can be categorized into five subtypes: *emotional*, *physical*, and *sexual abuse* and *emotional* and *physical neglect* (Bernstein et al., 2003).

Several studies have postulated the existence of a proportional relationship between the number of CM subtypes that children have experienced and the impoverishment of their mental health, with children who experience more CM subtypes having increased chances of presenting with mental health impoverishment (Cecil et al., 2017; Chapman et al., 2004).However, different types of trauma (such as abuse vs. neglect) may result in differential psychological and neurobiological impacts, due to differences in the nature of the trauma

* Corresponding author. Department of Dynamic and Clinical Psychology, Sapienza University of Rome, Italy.
E-mail addresses: valeria.carola@uniroma1.it, v.carola@hsantalucia.it (V. Carola).
[1] Equal contribution.

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(Teicher et al., 2016; Khan et al., 2015).For example, emotionally neglected children have been found to be socially withdrawn and inattentive (Erickson and Egeland, 2002) and demonstrate delays in their cognitive and social functions (Kaler and Freeman, 1994).Compared with physically abused and non-maltreated children, neglected children appear to have increased difficulty recognizing and discriminating between facial emotional expressions (Pollak et al., 2000). However, clinical studies that have attempted to investigate and dissect the effects of different CM subtypes (Teicher et al., 2016; Khoury et al., 2010) have had little success, likely because the co-occurrence of abuse and neglect is a common phenomenon (Chartier et al., 2010).

How CM and the different types of CM affect the development of psychopathologies in humans remain unanswered questions. Furthermore, the neurobiological mechanisms that are activated by exposure to different types of CM and their impacts on neurodevelopmental trajectories also remain unknown.

The difficulty dissecting the effects of different traumas can be partially overcome by using preclinical murine models. Our group demonstrated that exposure to different types of early-life stress (ELS) events during the same developmental stage in mice resulted in different behavioral and neurobiological outcomes during adulthood (Lo Iacono et al., 2015, 2017, 2018). In mice, during the third postnatal week, exposure to social isolation induced a depression-like phenotype during adulthood (Lo Iacono et al., 2015), whereas exposure to social stress, such as a threatening environment, resulted in increased susceptibility to cocaine-induced effects (Lo Iacono et al., 2018) during adulthood.

Here, we used these two murine models of ELS exposure to dissect the neurobiological consequences of exposures to different types of stressful emotional-social experiences at an early age. Inspired by clinical and preclinical evidence that strongly implicated the involvement of epigenetic mechanisms, such as DNA methylation (Murgatroyd et al., 2009) in the "translation" of ELS events into neurobiological and behavioral alterations during adulthood, we examined the global levels of DNA methylation in both brain and blood samples from mice that were exposed to different ELS events during the third postnatal week. DNA methylation is a repressive epigenetic marker, consisting of the covalent addition of a methyl group to the C-5 position of the cytosine ring in DNA by DNA methyltransferases (DNMTs). Dynamic DNA methylation changes occur during brain development and maturation, and the large-scale reconfiguration of the neuronal epigenome has been observed in the mouse brain during the third postnatal week (Lister et al., 2013).

Because different stressful experiences could differentially impact specific brain circuits, we examined the levels of global DNA methylation in subcortical regions that are involved in emotional, reward, and cognitive processes (Kruse et al., 2018; McEwen and Morrison, 2013), including the striatum, nucleus accumbens, amygdala, and hippocampus. Inspired by recent studies that have demonstrated key roles played by microglia during neurodevelopment and as sensors of early stressful environments (Catale et al., 2020; Paolicelli and Ferretti, 2017; Schafer et al., 2012), we also evaluated whether different types of ELS experiences impact the epigenetic programming and DNA methylation status of microglia in these brain areas (Catale et al., 2020; Paolicelli and Ferretti, 2017; Schafer et al., 2012).

Existing evidence supports the existence of intense cross-talk between the brain immune system (microglia) and peripheral blood immune cells (lymphocytes, monocytes, and macrophages) (Norris and Kipnis, 2019; Greenhalgh et al., 2020), which plays a relevant role in the induction of behavioral and psychological alterations following ELS exposure (Lo Iacono et al., 2018;Danese and Lewis, 2017). Therefore, we examined the consequences of ELS exposure on the DNA methylation/demethylation machinery in peripheral blood mononuclear cells (PBMCs).

Finally, we conducted a human clinical study to explore the possibility that exposure to childhood abuse (*sexual, physical, and emotional abuse*) or childhood neglect (*emotional and physical neglect*) may be associated with changes in the DNA methylation status of two clinical populations. Inspired by our preclinical behavioral data (Lo Iacono et al., 2015, 2018), we tested our hypothesis in PBMCs from a population of individuals who received cocaine use disorder (CUD) diagnoses and from subjects who received major depressive disorder (MDD) diagnoses. Overall, our preclinical findings strongly supported the differential impacts of the two examined ELS paradigms on the DNA methylation status in the brains and blood samples from mice. In humans, we observed a strong effect of the psychopathology and diagnosis on DNA methylation, but no effect of ELS exposure was identified.

2. Methods

2.1. Animals and breedings

Seven-week-old DBA2/J @Ico (DBA) male and female and CD1 male mice were purchased from Charles River Laboratories (Calco, Italy). For the production of pups, DBA/2J male and female mice were mated at 12 weeks of age and fathers were removed before parturition. Manipulation protocols were performed during the light cycle from 10:30 to 14:30. Mice were kept at constant temperature (21 ± 1 C) and humidity (55 ± 5%). Food and water were provided ad libitum, and mice were housed on a 12:12 light: dark cycle with lights on at 0700 h. All experiments were carried out in accordance with Italian national law (DL 26/2014) on the use of animals for research based on the European Communities Council Directive (2010/63/UE), and approved by the ethics committee of the Italian Ministry of Health (license/approval ID #: 42/2015-PR) and by local Ethical Committee of the Santa Lucia Foundation.

2.2. Juvenile stress procedure

Male and female mouse pups were assigned to control (CTR), Early Social Isolation (ESI) or Early Social Stress (ESS) group at postnatal day (PD) 14. In the CTR group mothers and offspring were left undisturbed until weaning. In the ESI group each pup was singly housed in a novel clean bedding cage for 30 min per day from PD14 to 21. In the ESS group, each pup was housed in a cage with a resident adult CD1 male mouse (different every day) for 30 min per day from PD14 to 21 (Lo Iacono et al., 2015, 2018). To avoid killing of the pups CD1 males were gonadectomized and single housed one month before to be used in the stress protocol. No cage cleaning was performed in the offspring cage from birth to weaning. All pups were weaned at PD22 and used for the biological analyses at 8–10 weeks of age. The experimental sample size was determined with the aid of online available software (http://www.stat.ubc.ca/~rollin/stats/ssize). To perform this calculation, we used values (e.g. means and common standard deviation) obtained from similar experiments previously performed in our laboratory.

2.3. Brain tissue isolation, DNA preparation, and global DNA methylation quantification

Mice (N = 7 mice/group, male/female, m/f, = 3/4) were sacrificed by decapitation in adulthood. Brains were dissected and stored at −80 °C. Punches of striatum, nucleus accumbens, hippocampus and amygdala were obtained from coronal brain slices no thicker than 300 μm according to Ventura et al (2002). DNA from punches was subsequently isolated using Genomic DNA purification Kit (Norgen Biotek, Thorold, Canada) according to the manufacturer instructions. DNA quantity was determined by absorbance at 280 nm using a NanoDrop UV-VIS spectrophotometer.

For global DNA methylation quantification, the Epigentek Methylflash Methylated DNA Quantification Kit (Colorimetric) was used according to manufacturer instructions. This Kit is based on ELISA technology, and utilizes optimized antibodies specific to 5-methylcytosine (5-mC) with no cross reactivity to unmethylated cytosine and negligible cross-reactivity to hydroxymethylcytosine. In this assay, DNA

is bound to strip wells; the methylated fraction is detected using capture and detection antibodies and then quantified by measuring absorbance via microplate reader (Molecular Devices M3, Sunnyvale, CA). According to the manufacturer instructions, a standard curve is built with the aid of DNA methylated at a known level (50%). The optical density measures of individual DNA samples are then interpolated with the curve and normalized to their total loading (100 ng) to obtain the percentage of methylation.

2.4. Immunofluorescence and confocal microscopy

At PD60, mice were sacrificed by decapitation, brains were removed, post-fixed in paraformaldehyde 4%, washed in PBS and transferred to a 30% sucrose solution at 4 °C until sinking (Lo Iacono et al., 2017). DNA strand was opened using the procedure described by Wossidlo and colleagues with minor modifications (Wossidlo et al., 2011). Briefly, 30 μm-thick transverse sections were treated for 30 min with 2 N HCl. Neutralization of the acidic solution was performed by 2 washing steps with Na3BO4, then slices were incubated for 30 min at room temperature (RT) with a blocking solution containing phosphate buffer (PB) + 1% bovine serum albumin. Afterwards, sections were incubated overnight at 4 °C in PB + 0.3% Triton X-100 with mouse anti-5-mC antibody (1:1000, #C15200081, Diagenode) for global DNA methylation staining (no cross reactivity to unmethylated cytosine and hydroxymethylcytosine) or with a cocktail of mouse anti-5-mC (1:1000) and rabbit anti-IBA-1 (1:700, #019–19741, Wako Lab Chemicals) antibodies for global DNA methylation and microglial cells staining. After 3 rinses in PB, sections were incubated for 2 h at RT with a cocktail of secondary antibodies including Alexa Fluor 555 conjugated donkey anti-mouse (#A-31570, 1:200; ThermoFisher Scientific) and Alexa Fluor 488 conjugated donkey anti-rabbit (#A-21206). To avoid staining variability, sections of ESI, ESS and CTR mice were concomitantly incubated with the same cocktail of primary and secondary antibodies. Sections were rinsed, DAPI-counterstained, mounted using an anti-fade medium (Fluoromount; Sigma, Milan, Italy), coverslipped and analyzed by a confocal laser scanning microscope (Zeiss CLSM800, Germany). Confocal acquisition of the areas of interest (dorsolateral and dorsomedial striatum, nucleus accumbens shell and core, basolateral and central amygdala, dorsal hippocampal CA1, CA3, dentate gyrus) was performed by using a 20x/0.50 objective (Plan-Apochromat, Zeiss; zoom factor = 0.5). The confocal images acquisitions were performed so that all samples were captured using consistent settings for laser power and detection gain. For final figures, images were exported in TIFF format and contrast and brightness were adjusted.

2.5. Densitometric analyses of fluorescence images

Quantitative analyses were performed off-line on confocal images acquired through the 20x objective. Expression levels of 5-mC in DAPI-stained nuclei were quantified as previously reported by Catanzaro et al. (2016). Briefly, after background subtraction, the 5-mC signal was quantified by manually outlining individual nuclei (within DAPI staining) and measuring cell associated fluorescence intensity with the ImageJ software (http://rsb.info.nih.gov/ij//; National Institutes of Health, Bethesda, MD). The F/A ratio defines mean fluorescence of individual cells (F) normalized to total nuclear area (A). For each subregion, the analysis was done on approximately 200 cells per group across three regularly spaced sections/animal (N = 3 mice/group, m/f = 2/1) and it was conducted blind to the animal's experimental group. For quantification of 5-mC expression levels in microglial cells, only DAPI-stained nuclei colocalizing with IBA-1 staining were considered, and approximately 100 cells per group across three regularly spaced sections/animal (N = 3 mice/group, m/f = 2/1) were analyzed. For quantification of microglial cells, IBA-1 labeled cells within each section were manually counted, as previously reported (Lo Iacono et al., 2018). Only immunolabeled cells with a distinct nucleus (DAPI-positive) in the focal plane were considered. Soma size of the IBA1-labeled cells was measured by manually outlining individual soma (Lo Iacono et al., 2018). Dentate gyrus has been excluded from microglia/5-mC analyses because the high compactness of cells within the granule layer of this structure made it difficult to clearly detect 5-mC signal in microglia nuclei.

2.6. PBMCs isolation, DNA and RNA purification

Mouse blood was collected in EDTA tubes when the mice were 80 days old (for RNA, CTR N = 9, m/f = 5/4; ESI N = 6, m/f = 3/3; ESS N = 8; m/f = 4/4; for DNA, CTR N = 7, m/f = 3/4; ESI N = 6, m/f = 4/3; ESS N = 8, m/f = 3/4). PBMCs were extracted from blood samples using Red Blood Cell (RBC) lysis buffer (Norgen Biotek) according to the manufacturer's protocol and RNA and DNA from different samples were subsequently isolated using Total RNA purification Plus Kit (Norgen Biotek) and Genomic DNA isolation kit (Norgen Biotek), respectively. RNA and DNA quantity were determined by absorbance at 260 and 280 nm respectively, using a NanoDrop UV-VIS spectrophotometer and RNA quality was assessed by the use of Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples were stored at −80 °C for further analysis.

2.7. Quantitative real time RT-PCR (qPCR)

cDNA was obtained using the High Capacity Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). cDNA templates (10 ng) were processed by qPCR using the 7900HT thermal cycler apparatus equipped with the SDS software version 2.3 (Applied Biosystems) for data collection. Taqman primer sets (Applied Biosystem, Taqman primers codes for Dnmt1: Mm01151063_m1; Dnmt3a: Mm00432881_m1; Dnmt3b: Mm01240113_m1; Tet1: Mm01169087_m1; Tet 3: Mm00805756_m1; Mecp2: Mm01193537_g1) were used for cDNA amplification and Ct values were normalized to the averaged Ct values of TATA box binding protein (Tbp) and phosphoglycerate kinase 1 (Pgk1) mRNA (Taqman primers codes for Tbp: Mm00446974_m1; Pgk1: Mm01225301_m1). All data were run in triplicate and analyzed using the ΔΔC(t) method (Schmittgen and Livak, 2008).

2.8. Human study

Participants included two clinical groups and one control group. The first clinical group was made up by 30 consecutive outpatients (8 men and 22 women) with a DSM-IV principal diagnosis of major depression (MDD) recruited from the adult psychiatric clinic of the University of Rome Tor Vergata. Patients were assessed with the Structured Clinical Interview for the DSM-IV (SCID-I) to establish axis I principal diagnosis. Structured interview was combined with the Mood Disorder Questionnaire (MDQ) to exclude current or past bipolar disorders (Wang et al., 2015). To be included in the clinical group, participants should have a history of recurrent depression (more than two episodes throughout their life), no comorbid axis I psychiatric disorders and an age between 18 and 65 years (mean +SE. = 50 ± 2 years). All patients were on treatment with SSRI antidepressant drugs (daily equivalent doses of 50–100 mg of sertraline) (Hayasaka et al., 2015).

The second clinical group was made up by 28 consecutive outpatients (25 men and 3 women) with a DSM-V principal diagnosis of cocaine use disorder (CUD) recruited from the Centers for Substance Abuse Treatment of Rome and Molise. To be included in the clinical group, participants should have a history of recurrent CUD and an age between 18 and 65 years (mean +SE. = 39 ± 1.5 years). No pharmacological treatment for CUD was provided to this group.

The healthy control group included 32 volunteers (18 men and 14 women; mean age +SE = 40 ± 1.5 years) who reported no symptoms of mental disorders based on screening questions focusing on most common DSM diagnoses.

The presence of acute or chronic inflammatory diseases and/or treatment with anti-inflammatory drugs were exclusion criteria for all participants. Obese people were not included in the study. Prior to enrolment, all participants were given a complete description of the study and signed a written informed consent. The study was approved by the Fondazione Santa Lucia (FSL) Intramural Ethical Committee.

2.9. Clinical assessment

The exposure to childhood abuse and neglect was measured using the Childhood Trauma Questionnaire (CTQ;Bernstein and Fink, 1998) in the entire sample. We used the short version (28 items) of the CTQ to assess the presence of emotional abuse, physical abuse, sexual abuse, emotional neglect, and physical neglect in our samples. Each scale of CTQ is presented in a 5-point Likert-type scale ranging from 5 to 25. Emotional abuse refers to verbal assaults on a child's sense of worth or well-being, or any humiliating or threatening behavior directed toward a child by an older person (patients with scores higher than 12 being considered as emotionally abused). Physical abuse refers to bodily assaults on a child by an older person that pose a risk of, or result in, injury (patients with scores higher than 9 being considered as physically abused). Sexual abuse refers to sexual contact or conduct between a child and an older person, including explicit coercion (patients with scores higher than 7 being considered as sexually abused). Emotional neglect refers to the failure of caretakers to provide basic psychological and emotional needs, such as love, encouragement, belonging and support (patients with scores higher than 14 being considered as emotionally neglected). Physical neglect refers to failure to provide basic physical needs including food, shelter, and safety (patients with scores higher than 9 being considered as physically neglected). Patients who reported scores higher than cutoffs (using the suggested cutoff scores byBernstein et al. 1998) on at least one scale among the CTQ scales used to evaluate exposure to childhood abuse (emotional, sexual, and physical abuse) were classified as *abused patients*. In parallel patients who reported scores higher than cutoffs on at least one scale among the CTQ scales used to evaluate exposure to childhood neglect (emotional and physical neglect) were classified as *neglected patients*.

2.10. Human PBMCs isolation and DNA purification

Blood (5 ml) was drawn on all participants, collected in EDTA vacutainer (BD, Toronto, Ontario, Canada), and PBMCs and DNA were isolated within few hours from collection as described for mouse studies. Global methylation quantification was performed by ELISA using the Epigentek Methylflash Methylated DNA Quantification Kit methyas described for mouse studies.

2.11. Statistical analysis

In the mouse study, all parameters were subjected to either one-way or nested-one (stress-nested animal) way analysis of variance (ANOVA). ANOVA was followed, in cases of significance ($P < 0.05$), by Duncan's test. In the human study, multiple regression analysis was used to estimate the contribution of clinical status to global DNA methylation levels over and above the contribution of childhood abuse/neglect exposure. Statistical analyses were carried out by Statistica software Version 12.0 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Different ELS experiences impact global DNA methylation in mouse subcortical brain areas

To determine whether the two examined ELS paradigms (Fig. 1 A) have long-term impacts on the epigenetic programming of the brain, the level of global DNA methylation was quantified in the brains of ESI, ESS, and CTR adult mice, using an enzyme-linked immunosorbent assay (ELISA; 5-mC immunoreactivity, Fig. 1 B). Because the two stressful experiences (ESI and ESS) could differentially impact specific brain circuits, multiple brain areas were investigated, including the striatum,

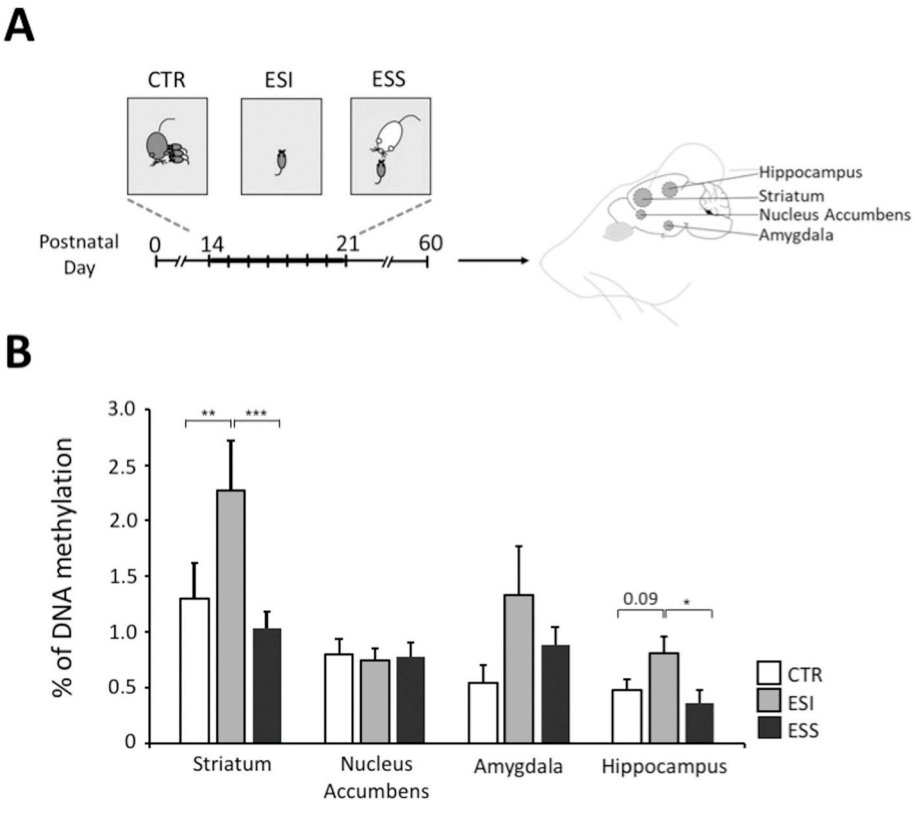


Fig. 1. Effects of Early Social Isolation or Early Social Stress on global DNA methylation levels in brain punches measured by ELISA. (**A**) Stress protocols applied from postnatal days (PDs) 14–21. Early Social Isolation (ESI) mice experienced daily (30′) isolation in a novel environment, whereas Early Social Stress (ESS) pups were exposed daily (30′) to an adult CD1. At PD60, animals were sacrificed, and brain punches were obtained. (**B**) Global DNA methylation level (expressed as % of DNA methylation) was quantified in brain punches of unhandled control (CTR), ESI and ESS adult mice by ELISA (N = 7 mice/group, m/f, = 3/4). ESI mice show higher global DNA methylation levels in the striatum, compared to both CTR and ESS mice. *P < 0.05, **P < 0.01, ***P < 0.001.

nucleus accumbens (NAc), amygdala, and hippocampus. We observed significantly increased global DNA methylation levels in the striatum in ESI mice compared with those in CTR and ESS mice (*stress effect*, F [2,18] = 3.601, P = 0.049). A similar effect was observed in the hippocampus (*stress effect*, F [2,18] = 3.612, P = 0.048), in which the global DNA methylation level was significantly increased in ESI mice compared with ESS mice. However, in this structure the global DNA methylation level only tended to be significantly higher in ESI mice than CTR mice, and no significant difference was observed between CTR and ESS mice. A similar trend was observed in the amygdala, although no significant differences were detected between groups. Notably, no changes in DNA methylation were observed for ESI or ESS mice in the nucleus accumbens using this approach.

3.2. Sub-regional and sub-cellular dissection of global DNA methylation modifications in the brain following different ELS experiences

To visualize the consequences of the two examined ESL paradigms on epigenetic programming at the sub-regional and sub-cellular levels, we detected global DNA methylation in different brain areas, by 5-mC immunostaining. The expression levels of 5-mC were measured in DAPI-immunostained cells in the dorsomedial (DM) and dorsolateral (DL) striatum, the core and shell of the NAc, the dentate gyrus (DG), Cornu Ammonis (CA) 1 and 3 of the hippocampus, and the basolateral (BLA) and central (CeA) amygdala. Consistent with the ELISA results, the expression level of 5-mC was significantly increased in ESI mice compared with those in ESS and CTR mice for all substructures (*stress-nested effect*: DM striatum, F [6222] = 3.629, P = 0.002; DL striatum, F [6232] = 61.323, P < 0.001; NAc core, F [6236] = 52.042, P < 0.001; NAc shell, F [6276] = 35.649, P < 0.001; DG, F [2221] = 13.271, P < 0.001; CA1, F [6224] = 42.908, P < 0.001; CA3, F [6235] = 16.920, P < 0.001; BLA, F [6226] = 17.827, P < 0.001; and CeA; F [6206] = 27.796, P < 0.001; Fig. 2). The only significant difference in 5-mC levels between ESS and CTR mice was observed in the DG (Fig. 2 D). These experiments showed stronger effects on global DNA methylation levels than for the ELISA-based assay, as shown in Fig. 1, likely due to the higher sensitivity of the immunofluorescence technique.

Recent evidence has demonstrated that the brain immune system (microglia) plays a role in the long-term behavioral and neurodevelopmental effects associated with ELS; therefore, we analyzed the 5-mC expression levels in ionized calcium-binding adapter molecule 1-positive (IBA-1+) microglia. In contrast with our observations in other cell populations, the levels of 5-mC in these cells were significantly lower in ESI mice compared with both ESS and CTR mice for all examined substructures (*animal-nested in stress effect*: DM striatum, F [6,99] = 38.707, P < 0.001; DL striatum, F [6110] = 34.158, P < 0.001; NAc core, F [6112] = 17.164, P < 0.001; NAc shell, F [6100] = 13.732, P < 0.001; CA1, F [6,87] = 3.825, P = 0.007; CA3, F [6,49] = 3.606, P = 0.012; BLA, F [6,80] = 6.910, P < 0.001; and CeA; F [6105] = 26.031, P < 0.001, Fig. 3B–E). A significant decrease in the 5-mC levels of IBA-1+ cells was observed for ESS mice compared with CTR mice but only in the CA1 region (Fig. 3 D). In contrast, the 5-mC levels of IBA-1+ microglia were significantly higher in ESS mice compared with ESI and CTR mice in the CA3 region and the BLA and CeA regions (Fig. 3 D, E).

To determine whether the epigenetic reprogramming in microglia was associated with altered microglial activity, we examined microglia morphology (soma size) and cell number (Fig. 3 F,G; figs1). We observed that microglia in the DL striatum, and core and shell of the NAc of ESI mice displayed significantly enlarged soma compared with those in CTR mice. In parallel, microglia in ESS mice showed significantly enlarged soma in the DL striatum compared with CTR mice (*animal-nested in stress effect:* DL striatum, F [6141] = 5.858, P = 0.010; NAc core, F [6133] = 10.022, P = 0.003; NAc shell, F [6148] = 3.475, P = 0.041; Fig. 3 F). Notably, the IBA1+ cell numbers were significantly increased in ESI mice compared with CTR mice in the CA1 and CA3 regions (Fig. 3 G). In parallel, IBA1+ cell numbers were also significantly increased in ESS mice compared with CTR mice in the CA3 region, although ESS mice had lower numbers of IBA-1+ cells than ESI and CTR mice in the BLA (*stress effect*: CA1, F [2,18] = 9.669, P < 0.001; CA3, F [2,18] = 11.855, P < 0.001; BLA, F [2,18] = 14.238, P < 0.001; Fig. 3 G).

3.3. Different ELS experiences impact the DNA methylation machinery in blood immune cells

Considering the intense crosstalk between microglia and peripheral blood immune cells (lymphocytes, monocytes, and macrophages), we investigated whether exposure to ESI or ESS induced alterations in the global DNA methylation status of blood cells. Global DNA methylation levels were quantified in PBMCs from ESI, ESS, and CTR adult mice by ELISA. No significant effect of ESI or ESS exposure was observed for this parameter (Fig. 4A). However, based on the comparison of the data in Figs. 1 and 2, the ELISA-based assay might not be sensitive enough to detect more subtle changes in DNA methylation profiles.

Therefore, because of the difficulty in performing immunofluorescence staining of PBMCs, we used an indirect approach, evaluating the expression levels of genes that are involved in DNA methylation and demethylation in PBMCs from mice. Significant changes in gene expression were observed for several of the genes. Overall, the strongest effects were observed in ESI mice, which had significantly decreased expression levels of the methyltransferases Dnmt1, Dnmt3a and Dnmt3b and the methylation reader Mecp2 and significantly greater levels of ten-eleven-translocation methylcytosine dioxygenase (Tet1) compared with CTR mice. Reduced expression levels of Dnmt3a, Dnmt3b, and Mecp2 were observed in ESS mice versus CTR mice (*stress effect*: Dnmt1, F [2,22] = 6.414, P = 0.007; Dnmt3a, F [2,22] = 14.778, P = < 0.001; Dnmt3b, F [2,22] = 8.803, P = 0.002; Tet1, F [2,22] = 6.266, P = 0.008; Mecp2, F [2,22] = 18.703, P < 0.001; Fig. 4B).

3.4. Blood global DNA methylation levels differ in distinct clinical samples

To determine whether ELS impacts the DNA methylation levels in humans and whether these impacts are specific to experienced traumatic events, we examined the association between exposure to childhood abuse or childhood neglect and blood DNA methylation status in two different clinical populations: MDD and CUD patients. We conducted a multiple linear regression analysis to ascertain if, after controlling for the confounding effect of exposure to childhood abuse or neglect, the diagnosis was a significant predictor of the global DNA methylation levels in the sample. The independent variables were the diagnostic group (coded as 1 for healthy control subjects, 2 for MDD patients, and 3 for CUD patients), the exposure to childhood abuse (coded as 1 for abused and 2 for no abused), and the exposure to childhood neglect (coded as 1 for neglected and 2 for no neglected). The dependent variable was the global DNA methylation level. The model was significant (F [2, 85] = 8.46, P < 0.001), explaining 13% (adjusted R2) of the variance. After controlling for the effects of the childhood abuse and neglect, the clinical diagnosis emerged as significant predictor of global DNA methylation levels (*diagnosis*, Beta = 0.25, t = 2.11, P = 0.037; *childhood abuse*, Beta = 0.04, t = 0.36, P = 0.72; *childhood neglect,* Beta = 0.017, t = 0.14, P = 0.89). We observed significantly lower global DNA methylation levels in MDD individuals compared with healthy control subjects and CUD subjects (Fig. 5).

4. Discussion

Our findings showed that two different types of ELS experiences, which have been associated with depression-like (ESI) or addiction-like (ESS) behavior in adult mice, induce diverse and permanent alterations in DNA methylation in brain and blood samples. The direction and magnitude of these effects depend on the tissue (brain/blood), the brain structure, and the cellular population. Crucially, mice that are exposed to ESI, which mimics a condition of maternal neglect and isolation from

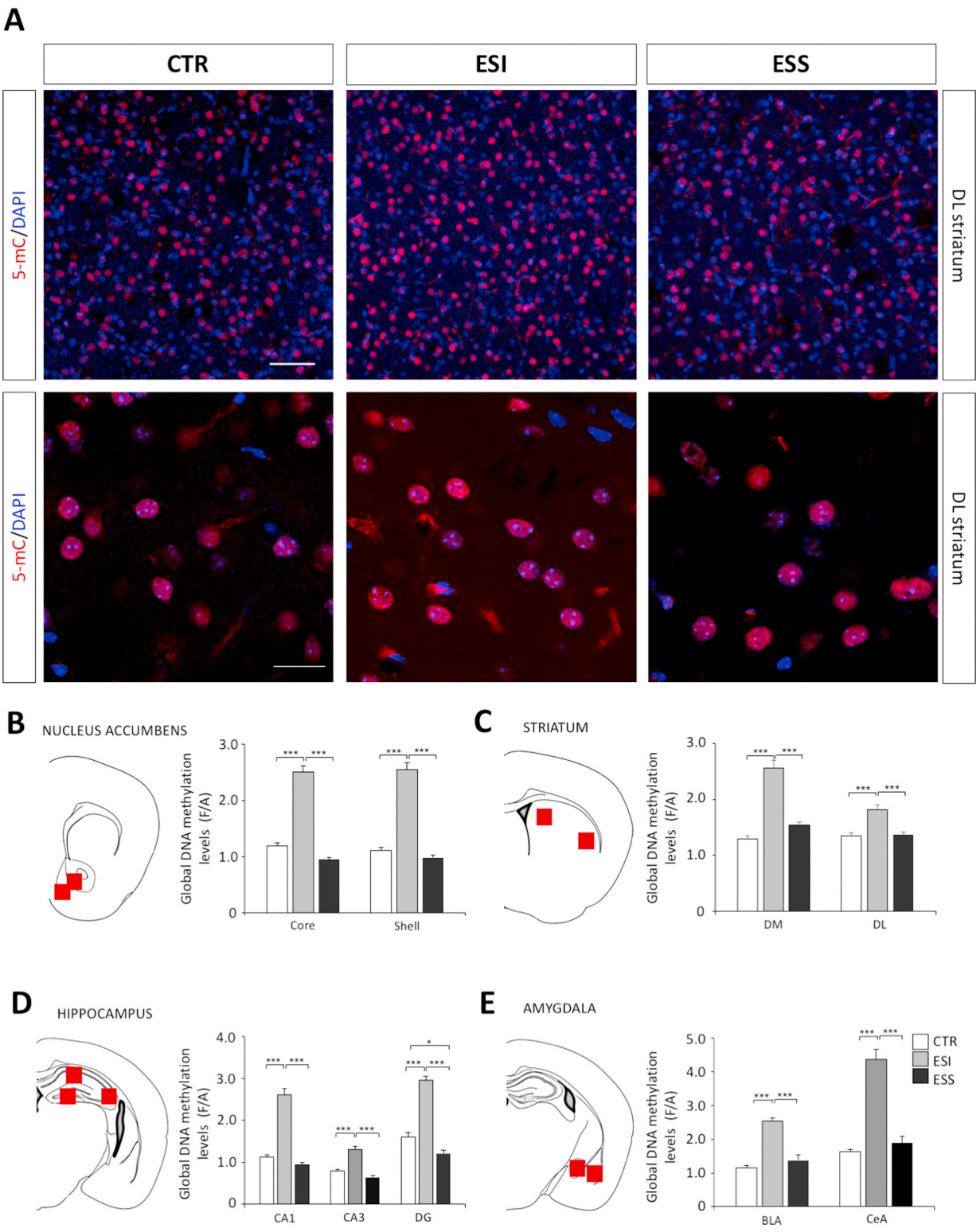
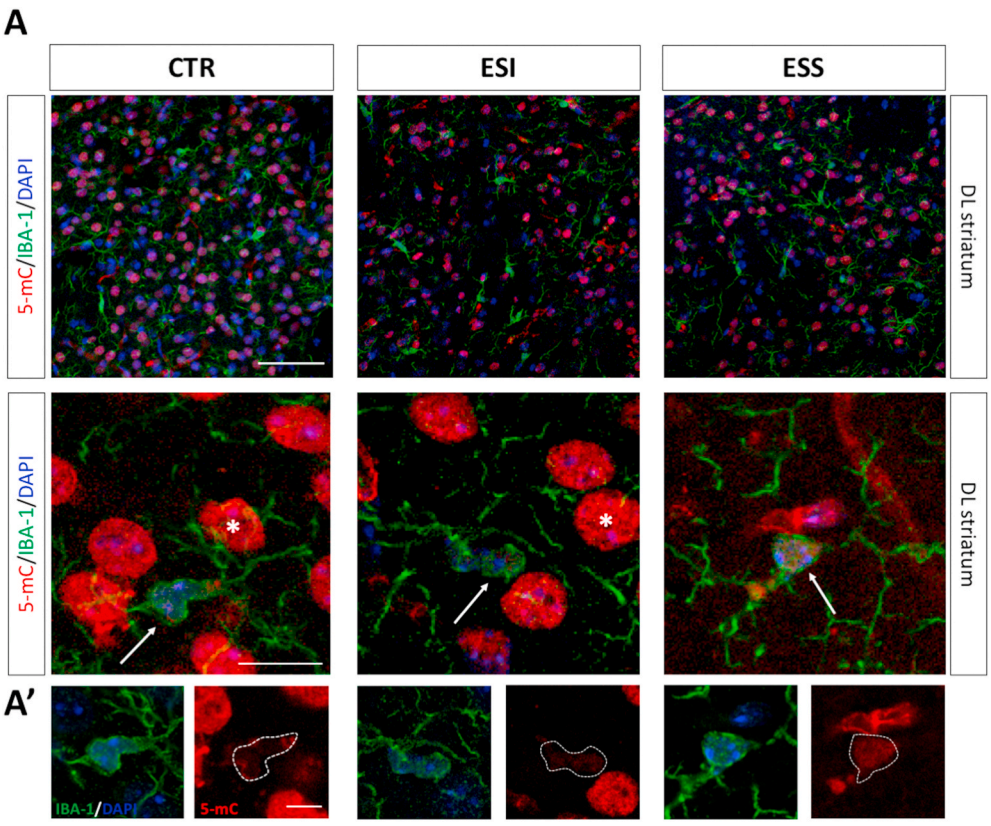


Fig. 2. Effects of ESI or ESS on global DNA methylation levels in DAPI + cells in different brain structures measured by immunohistochemistry. (**A**) Representative confocal images of 5-methylcytosine (5-mC) nuclear immunostaining for global DNA methylation in the dorsolateral (DL) striatum of CTR, ESI and ESS adult mice (N = 5 mice/group, m/f = 3/2) at lower (top) and higher (bottom) magnification. (**B-E**) Left: Schematic hemisection pictures showing localization of the areas analyzed (red square boxes). Right: quantification of global DNA methylation levels, expressed as mean 5-mC fluorescence of individual cells (F) normalized to total nuclear area (A). ESI mice show significantly higher global DNA methylation (F/A) in all the sub-structures analyzed, compared to both CTR and ESS mice. ESS mice show significantly reduced global DNA methylation in the dentate gyrus (DG), with respect to CTR. Scale bar (**A**): top 50 μm; bottom 20 μm *P < 0.05, ***P < 0.001. *Abbreviations*: dorsomedial, DM; cornu ammonis 1 and 3, CA1 and CA3; basolateral and central amygdala, BLA and CeA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



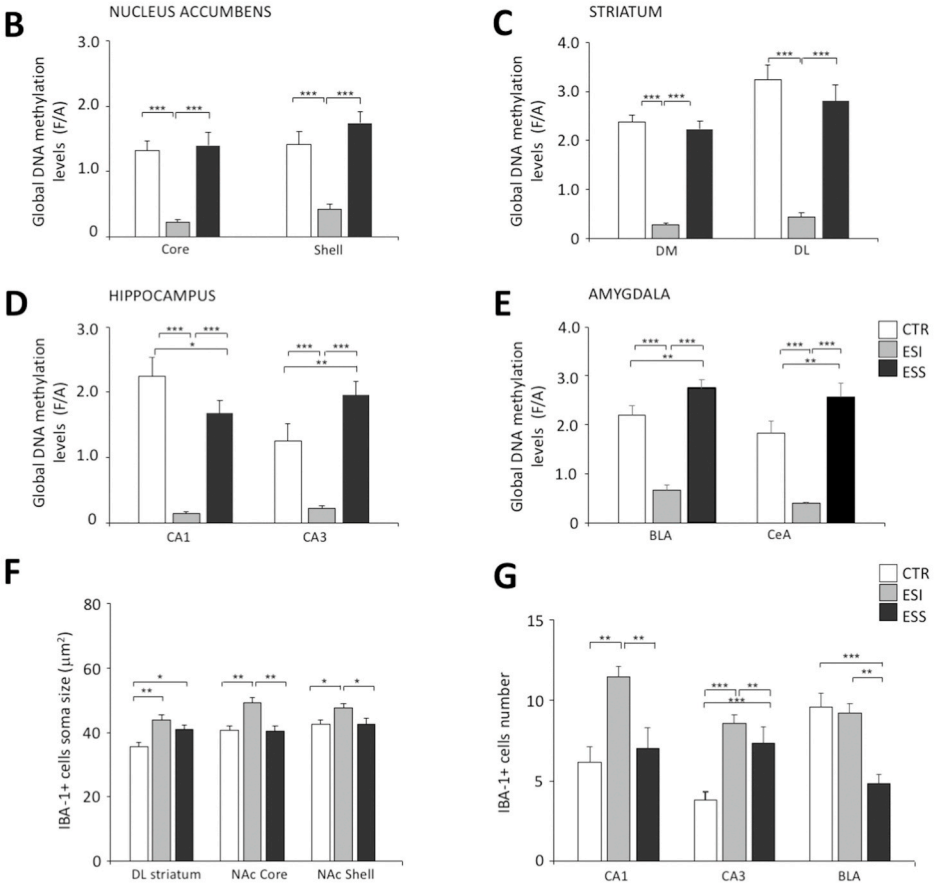


Fig. 3. Effects of ESI and ESS on global DNA methylation and activation of IBA-1+ cells (microglia) in different brain structures measured by immunohistochemistry. (**A**) Representative confocal images of 5-mC and IBA-1 immunostaining in the DL striatum of CTR, ESI and ESS adult mice at lower (top) and higher (bottom) magnification. Arrows indicate microglial cell bodies; asterisks indicate 5-mC immunostained non-microglial nuclei. (**A′**) Split channels images showing microglial cell bodies (DAPI/IBA-1+) and 5-mC signal in the different groups. (**B-E**) Quantification of global DNA methylation levels (F/A) in colocalizing 5-mC/IBA-1 immunostained nuclei across different brain regions. Microglia from ESI mice show significantly lower global DNA methylation levels in all the structures analyzed, compared to both CTR and ESS mice. Microglia from ESS mice show a significant increase in global DNA methylation than CTR in the CA3, BLA and CeA, and a significant decrease in the CA1. (**F**) Soma size of IBA-1+ cells was increased in the DL striatum, and nucleus accumbens (NAc) shell and core of ESI mice. ESS mice show a slight increase in the soma size of IBA-1+ cells in the DL striatum. (**G**) Quantification of IBA-1+ cells revealed an increase in microglial cell number in the CA1 and CA3 subregions of ESI mice with respect to CTR and ESS, and a decrease in the BLA of ESS mice. Scale bar (**A**): top 50 μm; bottom 10 μm; (**A′**): 5 μm *P < 0.05, **P < 0.01, ***P < 0.001.

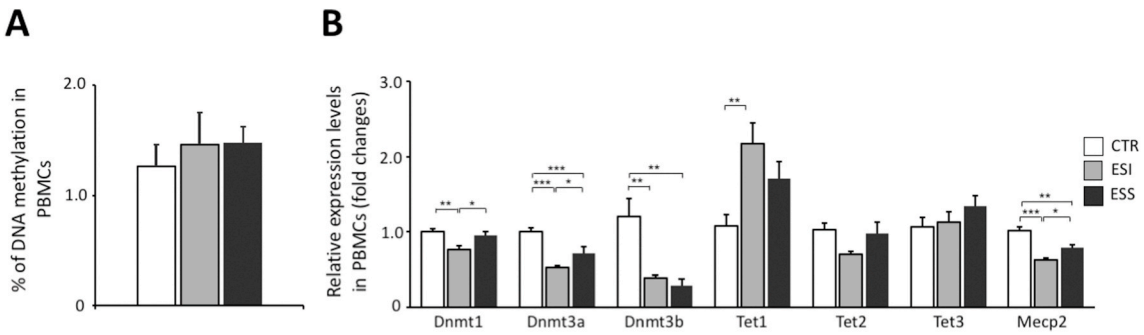


Fig. 4. Effects of ESI and ESS on global DNA Methylation levels and machinery in peripheral blood immune cells (PBMCs). (**A**) Global DNA methylation level (expressed as % of DNA methylation) was quantified in PBMCs of CTR, ESI and ESS adult mice (CTR N = 7, m/f = 3/4; ESI N = 6, m/f = 4/3; ESS N = 8, m/f = 3/4) by ELISA. No significant difference was detected. (**B**) Expression of genes belonging to the DNA methylation machinery was assessed in PBMCs of CTR, ESI and ESS adult mice (CTR N = 9, m/f = 5/4; ESI N = 6, m/f = 3/3; ESS N = 8; m/f = 4/4) by qPCR. ESI mice showed significant lower expression levels of Dnmt1, Dnmt3a, Dnmt3b, and Mecp2 genes and higher expression levels of Tet1 gene than CTR. Similarly, expression levels of Dnmt3a, Dnmt3b, and Mecp2 genes were reduced in ESS mice compared with CTR. Finally, ESI mice had significantly lower levels of Dnmt1, Dnmt3a, and Mecp2 than ESS mice. *P < 0.05, **P < 0.01, ***P < 0.001.

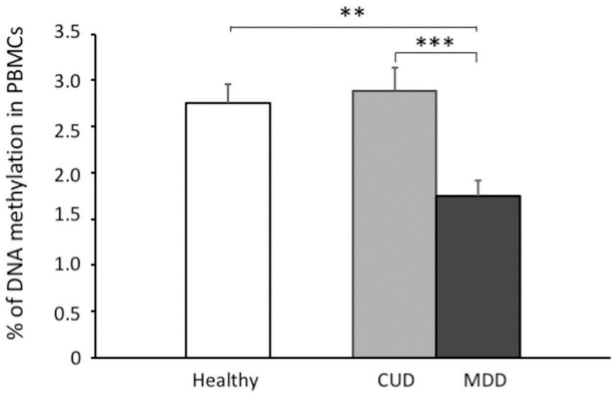


Fig. 5. Global DNA methylation levels in PBMCs from major depressive disorder (MDD) and cocaine use disorder (CUD) patients. (**A**) Global DNA methylation level (expressed as % of DNA methylation) was quantified in PBMCs of healthy subjects (healthy), MDD and CUD patients by ELISA. A significant effect of the diagnosis was observed, with MDD patients showing lower levels of global DNA methylation than CUD patients and healthy subjects. No difference between CUD patients and CTR was detected. **P < 0.01, ***P < 0.001.

peers, demonstrated more drastic and widespread changes compared with CTR and ESS mice. Within the brain, a general and substantial increase in global DNA methylation levels was detected in the striatum (in both the DL and DM subregions), NAc (core and shell), amygdala (BLA and CeA), and hippocampus (CA1, CA3, and DG) of ESI mice compared with CTR and ESS mice. In contrast, mice that were exposed to ESS, which mimics a condition of emotional abuse and exposure to a threatening environment, had lower global methylation levels only in the DG compared with CTR, although they had lower global methylation in all brain structures analyzed compared to ESI mice.

These results suggest that ESI induces more drastic epigenetic reprogramming than ESS in several brain structures, possibly leading to the negative regulation of specific transcriptional programs. Previous preclinical studies have demonstrated DNA methylation modulation following stressful maternal-pup interactions (Burns et al., 2018); however, in contrast with our study, previous studies examined these alterations in fewer brain regions or described these ELS-induced effects as being region-specific. Most of these studies focused on the hippocampus, because this brain region is targeted by the hypothalamic-pituitary axis (stress response mechanism), and they reported dramatic and lasting increases in global DNA methylation levels after exposure to maternal separation (McCoy et al., 2016) or maternal "maltreatment." associated with pup isolation (Doherty et al., 2016). Increased hippocampal Dnmt1 levels have been correlated with global DNA methylation levels (Brown et al., 2008) and were also detected in the adult offspring of low-care rat mothers (Zhang et al., 2010). A subtle but significant decrease in global DNA methylation levels, accompanied by increased methylation at specific sites and the upregulation of DNA methylation genes (including Dnmt1, 3a, and 3b), was observed in the NAc core of maternally separated rats during adulthood (Anier et al., 2014).

Compared with the results of these previous studies, our study investigated not only the effects of ELS on DNA methylation across a wide range of brain structures but also the effects of different ELS experiences on DNA methylation, bringing a notable advancement to the knowledge base of this field. The stronger effects for ESI compared with ESS suggest that experiencing significant reductions in social contacts during a developmental stage may be more critical for the "construction" of social behavior and sociability than experiencing social adversity (ESS), because ESI induces not only a depression-like phenotype (Lo Iacono et al., 2015) but also dramatically changes the epigenetic programming of multiple brain circuits. These results agree with recent evidence published for humans, for whom the strong impacts of parental psychological neglect and peer isolation on adolescent depression have been described (Christ et al., 2017).

Given the emerging roles of microglia in shaping brain circuits during development (Paolicelli and Ferretti, 2017; Schafer et al., 2012) and mediating the ELS-induced effects on brain circuitry and behavior (Catale et al., 2020; Cheray and Joseph, 2018), we decided to analyze whether and how ESI and ESS exposure impacts microglial DNA methylation. Unexpectedly, despite the observed increase in global DNA methylation in the brains of ESI mice, a drastic decrease in the global DNA methylation of microglia was observed for ESI mice compared with the other groups for all subregions. These data show that the epigenetic reprogramming that is induced by ESI is cell-specific, with opposite effects in microglia and non-microglia cells, namely neurons, astrocytes and oligodendrocytes. Consistent with the strong influence of ESI on microglia fate/programming, alterations in microglial soma sizes (a morphological parameter that is generally considered to be associated with altered microglial reactivity) were detected in NAc, and striatum (DL), whereas alterations in microglial numbers were only observed in the hippocampus (CA1 and CA3). Overall, the decreased methylation in microglia, accompanied by altered phenotypic expression, could represent a mechanism through which microglia retain memories of the ESI experience, becoming more reactive. This priming state is characterized by a transcriptional rearrangement and the expression of usually unexpressed genes (Perry and Holmes, 2014). Several reports have described hippocampal microglia as being extremely sensitive to ELS

(Catale et al., 2020; Niraula et al., 2017), and microglial changes in the NAc and striatum have been observed after maternal separation (Catale et al., 2020; Schwarz et al., 2011). Moreover, the pharmacological inhibition of ELS-induced microglial activation has been shown to rescue the ELS-induced depressive-like phenotype in adulthood (Catale et al., 2020; Gong et al., 2018; Wang et al., 2017).

Notably, exposure to ESS appears to have stronger effects in microglia than in non-microglia cells, especially in the amygdala, in which increased microglial methylation has been reported in ESS mice compared with CTR. Increased microglial methylation was accompanied by decreased microglial cell numbers in the BLA. Moreover, ESS induces greater microglial methylation in the CA3 and decreased microglial methylation in the CA1, confirming the diverse sensitivity of hippocampal subregions to ELS (Brown et al., 2008). Overall, these results, with previous evidence of the lasting modulation of microglial function in the ventral tegmental area after ESS (Lo Iacono et al., 2018), suggest that the experience of an adverse social environment during preadolescence induces focal effects (in specific target brain regions), specifically for the microglial population. In light of previous evidence that microglia contribute to the shaping of brain circuits during pre- and postnatal development (Paolicelli and Ferretti, 2017; Schafer et al., 2012) and that the pharmacological inhibition of ESS-induced microglial activation can rescue behavioral and brain circuitry functional alterations (Lo Iacono et al., 2018), we hypothesize that some of the phenotypic effects that are observed after ESS are microglia-mediated. Further studies that are designed to better understand how microglia react to ESS and how the microglial activity is modulated during the shaping of brain circuitry in response to ESS are, therefore, necessary to elucidate the mechanisms through which this type of ELS produces long-lasting effects.

Considering the intense cross-talk that exists between the microglia and peripheral immune cells to modulate the psychological abnormalities that are observed after ELS (Lo Iacono et al., 2018;Danese and Lewis, 2017), we evaluated whether the ELS-induced effects on DNA methylation in the brain could also be detected in the periphery by examining PBMCs. Whereas no effect of ELS on global DNA methylation in PBMCs was detected by ELISA, this result may be due to the low sensitivity of the experimental approach, as suggested by the results in Figs. 1 and 2, using ELISA and 5-mC immunofluorescence detection techniques, respectively. Notably, a significant effect of ELS on the expression of enzymes that are associated with DNA methylation and demethylation was observed. Exposure to ESI again induced stronger effects compared with ESS, with ESI mice showing expression changes in 5 of 7 genes (Dnmt1, Dnmt3a, Dnmt3b, Mecp2, and Tet1), whereas ESS mice showed expression changes in only 3 genes (Dnmt3a, Dnmt3b and Mecp2). These gene expression profiles suggest a general decrease in DNA methylation levels as suggested by previous reports (Kohli and Zhang, 2013; Goll and Bestor, 2005). Unfortunately, the low sensitivity of ELISA did not allow us to detect these changes through direct methods. Few preclinical studies have evaluated global DNA methylation levels in the blood after ELS experiences. In macaques, exposure to ELS (variable foraging demand stress) generally did not alter global DNA methylation levels, although increased global methylation was associated with increased stress reactivity (Kinnally et al., 2011). In rodents, ELS effects on DNA methylation in the blood were investigated by a candidate gene approach. For example, Beery et al. (2016) showed that exposure to "low" maternal care resulted in decreased methylation status in the oxytocin receptor locus in the blood. However, no changes in the methylation status of this gene were detected in the brain, suggesting that the observed peripheral epigenetic alteration is a useful biomarker for ELS exposure but may not necessarily reflect alterations in the brain (Beery et al., 2016). In our study, the effects of the two ELS experiences were observed in the blood, and these alterations were consistent with the changes in DNA methylation of microglia, rather than with the changes in other brain cells populations. This result could help explain why several preclinical and human studies (Bakulski et al., 2016; Walton et al., 2016) have failed to identify any correspondence between DNA methylation alterations in the blood and those in the brain. The alterations in PBMCs may parallel the changes in microglia; however, most studies examine methylation by studying whole-brain tissue, without distinguishing between neurons and glia, which is unable to reflect the immune changes in blood.

Our clinical results demonstrate an effect of the diagnosis, rather than an effect of the childhood abuse/neglect experience, with MDD patients showing decreased global DNA methylation in PBMCs compared with healthy individuals and CUD patients. This result is consistent with the large body of literature on alterations in DNA methylation on the genome-wide and gene-specific levels (Clark et al., 2019; Zhu et al., 2019) in MDD patients.

Many rodent studies have demonstrated a modulatory role for DNA methylation in cocaine-induced behaviors, through the pharmacological manipulation of this parameter (Anier et al., 2013; Han et al., 2010). Moreover, alterations in the expression and function of DNMTs have been described in numerous cocaine paradigms and in brain reward pathways (Vaillancourt et al., 2017). Although the effects of nicotine and ethanol on DNA methylation machinery have been repeatedly evaluated in clinical blood samples, no preclinical or clinical studies have investigated the effects of cocaine on blood DNA methylation. In this study, for the first time, DNA methylation levels were analyzed in CUD patients, but no effect of this substance was detected. However, in this study, the failure to detect an effect of CUD and ELS in human PBMCs might have been due to the poor sensitivity of the ELISA analysis.

Our clinical study has three main limitations: 1. DNA methylation was measured in MDD patients during antidepressant treatment, recent evidences suggest a modulatory effect of antidepressants on DNA methylation (Webb et al., 2020), thus we cannot rule out the possibility that our observations may be driven by the pharmacological treatment rather than by MDD diagnosis; 2. The clinical groups had a different male-female distribution due to the sex-specific incidence of these disorders, and finally 3. Potential differences in blood cell composition across clinical groups were not evaluated. Future studies on bigger scale, that possibly take into account the pharmacological treatments and in more sex-balanced groups, are needed to corroborate our results and evaluate if blood cell composition contributes to the reported biological effects.

Overall, our findings strongly support the hypothesis that different ELS experiences produce different behavioral outcomes through differential impacts on gene reprogramming in the brain. Therefore, considering the impacts of ELS experiences on child psychology to be proportional would appear to be reductive. Instead, our study strongly indicates that the type and quality of early-life traumatic events are strongly relevant to modulating the susceptibility to specific psychopathological outcomes.

Considering the evidence that ESI effects are widespread and strongly involve DNA methylation, developing a systematic drug that targets this mechanism is a potentially new pharmacological approach. Consequently, whether such a pharmacological approach to DNA methylation would be able to counteract or mitigate abnormal adult phenotype should be investigated, opening a new avenue of treatment for the psychological and pathological outcomes that are associated with early-life neglect.

Author statement contributors

Clarissa Catale: Contributed to perform the biological experiments, to interpret the results and to write the manuscript.

Silvia Bussone: Contributed to perform the biological experiments and to interpret the results.

Luisa Lo Iacono: Contributed to perform the biological experiments and to interpret the results.

Maria Teresa Viscomi: Contributed to perform the immunohistochemistry experiments and prepare figures.

Daniela Palacios: Formal analysis.

Alfonso Troisi: In charge of patients 'recruitment and clinical assessment. Contributed to interpret human results.

Valeria Carola: Formal analysis, designed the study and wrote the manuscript.

All authors have read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2020.100249.

References

Anier, K., Malinovskaja-Gomez, K., Pruus, K., Aonurm-Helm, A., 2014. Maternal separation is associated with DNA methylation and behavioural changes in adult rats. Eur. Neuropsychopharmacol 24, 459–468. https://doi:10.1016/j.euroneuro.2013.07.012.

Anier, K., Zharkovsky, A., Kalda, A., 2013. S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice. Int. J. Neuropsychopharmacol. 16, 2053–2066. https://doi:10.1017/S1461145713000394.

Bakulski, K.M., Halladay, A., Hu, V.W., Mill, J., Fallin, M.D., 2016. Epigenetic research in neuropsychiatric disorders: the "tissue issue. Curr. Behav. Neurosci. Rep. 3, 264–274. https://doi:10.1007/s40473-016-0083-4.

Beery, A.K., McEwen, L.M., MacIsaac, J.L., Francis, D.D., Kobor, M.S., 2016. Natural variation in maternal care and cross-tissue patterns of oxytocin receptor gene methylation in rats. Horm. Behav. 77, 42–52. https://doi:10.1016/j.yhbeh.2015.05.022.

Bernstein, D.P., Fink, L., 1998. Childhood Trauma Questionnaire : a Retrospective Self-Report. The Psychological Corporation, San Antonio, TX.

Bernstein, D.P., Stein, J.A., Newcomb, M.D., Walker, E., Pogge, D., Ahluvalia, T., et al., 2003. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child Abuse Negl. 27, 169–190. https://doi:10.1016/s0145-2134(02)00541-0.

Bremne, J.D., Vermetten, E., 2001. Stress and development: behavioral and biological consequences. Dev. Psychopathol. 13, 473–489. https://doi:10.1017/s0954579401003042.

Brown, S.E., Weaver, I.C., Meaney, M.J., Szyf, M., 2008. Regional-specific global cytosine methylation and DNA methyltransferase expression in the adult rat hippocampus. Neurosci. Lett. 440, 49–53. https://doi:10.1016/j.neulet.2008.05.028.

Burns, S.B., Szyszkowicz, J.K., Luheshi, G.N., Lutz, P.E., Turecki, G., 2018. Plasticity of the epigenome during early-life stress. Semin. Cell Dev. Biol. 77, 115–132. https://doi:10.1016/j.semcdb.2017.09.033.

Catale, C., Gironda, S., Lo Iacono, L., Carola, V., 2020. Microglial function in the effects of early-life stress on brain and behavioral development. J. Clin. Med. 9. https://doi:10.3390/jcm9020468.

Catanzaro, G., Pucci, M., Viscomi, M.T., Lanuti, M., Feole, M., Angeletti, S., et al., 2016. Epigenetic modifications of Dexras 1 along the nNOS pathway in an animal model of multiple sclerosis. J. Neuroimmunol. 294, 32–40. https://doi:10.1016/j.jneuroim.2016.03.009.

Cecil, C.A., Viding, E., Fearon, P., Glaser, D., McCrory, E.J., 2017. Disentangling the mental health impact of childhood abuse and neglect. Child Abuse Negl. 63, 106–119. https://doi:10.1016/j.chiabu.2016.11.024.

Chapman, D.P., Whitfield, L., Felitti, V.J., Dube, S.R., Edwards, V.J., Anda, R.F., 2004. Adverse childhood experiences and the risk of depressive disorders in adulthood. J. Affect. Disord. 82, 217–225. https://doi:10.1016/j.jad.2003.12.013.

Chartier, M.J., Walker, J.R., Naimark, B., 2010. Separate and cumulative effects of adverse childhood experiences in predicting adult health and health care utilization. Child Abuse Negl. 34, 454–464. https://doi:10.1016/j.chiabu.2009.09.020.

Cheray, M., Joseph, B., 2018. Epigenetics control microglia plasticity. Front. Cell. Neurosci. 12, 243. https://doi:10.3389/fncel.2018.00243.

Christ, S.L., Kwak, Y.Y., Lu, T., 2017. The joint impact of parental psychological neglect and peer isolation on adolescents' depression. Child Abuse Negl. 69, 151–162. https://doi:10.1016/j.chiabu.2017.04.015.

Clark, S.L., Hattab, M.W., Chan, R.F., Shabalin, A.A., Han, L.K.M., Zhao, M., et al., 2019. A methylation study of long-term depression risk. Mol. Psychiatr. 25, 1334–1343. https://doi:10.1038/s41380-019-0516-z.

Danese, A., Lewis, S.J., 2017. Psychoneuroimmunology of early-life stress: the hidden wounds of childhood trauma? Neuropsychopharmacology 42, 99–114. https://doi:10.1038/npp.2016.198.

Doherty, T.S., Forster, A., Roth, T.L., 2016. Global and gene-specific DNA methylation alterations in the adolescent amygdala and hippocampus in an animal model of caregiver maltreatment. Behav. Brain Res. 298, 55–61. https://doi:10.1016/j.bbr.2015.05.028.

Erickson, M.F., Egeland, B., 2002. Child Neglect. In: Myers, J.E.B., Berliner, L., Briere, J., Hendrix, C.T., Jenny, C., Reid, T.A. (Eds.), The APSAC Handbook on Child Maltreatment. Sage Publications, Inc, pp. 3–20.

Goll, M.G., Bestor, T.H., 2005. Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. 74, 481–514. https://doi:10.1146/annurev.biochem.74.010904.153721.

Gong, Y., Tong, 1, Yang, R., Hu, W., Xu, X., Wang, W., et al., 2018. Dynamic changes in hippocampal microglia contribute to depressive-like behavior induced by early social isolation. Neuropharmacology 135, 223–233. https://doi:10.1016/j.neuropharm.2018.03.023.

Greenhalgh, A.D., David, S., Bennett, F.C., 2020. Immune cell regulation of glia during CNS injury and disease. Nat. Rev. Neurosci. 21 (3), 139–152. https://doi.org/10.1038/s41583-020-0263-9.

Han, J., Li, Y., Wang, D., Wei, C., Yang, X., Sui, N., 2010. Effect of 5-aza-2-deoxycytidine microinjecting into hippocampus and prelimbic cortex on acquisition and retrieval of cocaine-induced place preference in C57BL/6 mice. Eur. J. Pharmacol. 642, 93–98. https://doi:10.1016/j.ejphar.2010.05.050.

Hayasaka, Y., Purgato, M., Magni, L.R., Ogawa, Y., Takeshima, N., Cipriani, A., et al., 2015. Dose equivalents of antidepressants: evidence-based recommendations from randomized controlled trials. J. Affect. Disord. 180, 179–184. https://doi:10.1016/j.jad.2015.03.021.

Kaler, S.R., Freeman, B.J., 1994. Analysis of environmental deprivation: cognitive and social development in Romanian orphans. JCPP (J. Child Psychol. Psychiatry) 35, 769–781. https://doi:10.1111/j.1469-7610.1994.tb01220.x.

Khan, A., McCormack, H.C., Bolger, E.A., McGreenery, C.E., Vitaliano, G., Polcari, A., Teicher, M.H., 2015. Childhood maltreatment, depression, and suicidal ideation: critical importance of parental and peer emotional abuse during developmental sensitive periods in males and females. Front. Psychiatr. 6, 42. https://doi:10.3389/fpsyt.2015.00042.

Khoury, L., Tang, Y.L., Bradley, B., Cubells, J.F., Ressler, K.J., 2010. Substance use, childhood traumatic experience, and Posttraumatic Stress Disorder in an urban civilian population. Depress. Anxiety 27, 1077–1086. https://doi:10.1002/da.20751.

Kinnally, E.L., Feinberg, C., Kim, D., Ferguson, K., Leibel, R., Coplan, J.D., Mann, J.J., 2011. DNA methylation as a risk factor in the effects of early life stress. Brain Behav. Immun. 25, 1548–1553. https://doi:10.1016/j.bbi.2011.05.001.

Kohli, R.M., Zhang, Y., 2013. TET enzymes, TDG and the dynamics of DNA demethylation. Nature 502, 472–479. https://doi:10.1038/nature12750.

Kruse, O., Tapia Leon, I., Stalder, T., Stark, R., Klucken, T., 2018. Altered reward learning and hippocampal connectivity following psychosocial stress. Neuroimage 171, 15–25. https://doi:10.1016/j.neuroimage.2017.12.076.

Lister, R., Mukamel, E.A., Nery, J.R., Urich, M., Puddifoot, C.A., Johnson, N.D., et al., 2013. Global epigenomic reconfiguration during mammalian brain development. Science 341 (6146). https://doi.org/10.1126/science.1237905, 1237905.

Lo Iacono, L., Catale, C., Martini, A., Valzania, A., Viscomi, M.T., Chiurchiù, V., et al., 2018. From traumatic childhood to cocaine abuse: the critical function of the immune system. Biol. Psychiatr. 84, 905–916. https://doi:10.1016/j.biopsych.2018.05.022.

Lo Iacono, L., Valzania, A., Visco-Comandini, F., Aricò, E., Viscomi, M.T., Castiello, L., et al., 2017. Social threat exposure in juvenile mice promotes cocaine-seeking by altering blood clotting and brain vasculature. Addiction Biol. 22, 911–922. https://doi:10.1111/adb.12373.

Lo Iacono, L., Visco-Comandini, F., Valzania, A., Viscomi, M.T., Coviello, M., Giampà, A., et al., 2015. Adversity in childhood and depression: linked through SIRT1. Transl. Psychiatry 5, e629. https://doi:10.1038/tp.2015.125.

McCoy, C.R., Rana, S., Stringfellow, S.A., Day, J.J., Wyss, J.M., Clinton, S.M., Kerman, I.A., 2016. Neonatal maternal separation stress elicits lasting DNA methylation changes in the hippocampus of stress-reactive Wistar Kyoto rats. Eur. J. Neurosci. 44, 2829–2845. https://doi:10.1111/ejn.13404.

McEwen, B.S., Morrison, J.H., 2013. The brain on stress: vulnerability and plasticity of the prefrontal cortex over the life course. Neuron 79, 16–29. https://doi:10.1016/j.neuron.2013.06.028.

Murgatroyd, C., Patchev, A.V., Wu, Y., Micale, V., Bockmühl, Y., Fischer, D., et al., 2009. Dynamic DNA methylation programs persistent adverse effects of early-life stress. Nat. Neurosci. 12, 1559–1566. https://doi:10.1038/nn.2436.

Niraula, A., Sheridan, J.F., Godbout, J.P., 2017. Microglia priming with aging and stress. Neuropsychopharmacology 42, 318–333. https://doi:10.1038/npp.2016.185.

Norris, G.T., Kipnis, J., 2019. Immune cells and CNS physiology: microglia and beyond. J. Exp. Med. 216 (1), 60–70. https://doi.org/10.1084/jem.20180199.

Paolicelli, R.C., Ferretti, M.T., 2017. Function and dysfunction of microglia during brain development: consequences for synapses and neural circuits. Front. Synaptic Neurosci. 9, 9. https://doi:10.3389/fnsyn.2017.00009.

Perry, V.H., Holmes, C., 2014. Microglial priming in neurodegenerative disease. Nat. Rev. Neurol. 10, 217–224. https://doi:10.1038/nrneurol.2014.38.

Pollak, S.D., Cicchetti, D., Hornung, K., Reed, A., 2000. Recognizing emotion in faces: developmental effects of child abuse and neglect. Dev. Psychol. 36, 679–688. https://doi:10.1037/0012-1649.36.5.679.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., et al., 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74, 691–705. https://doi:10.1016/j.neuron.2012.03.026.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3, 1101–1108. https://doi:10.1038/nprot.2008.73.

Schwarz, J.M., Hutchinson, M.R., Bilbo, S.D., 2011. Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. J. Neurosci. 31, 17835–17847. https://doi:10.1523/JNEUROSCI.3297-11.2011.

Teicher, M.H., Samson, J.A., 2013. Childhood maltreatment and psychopathology: a case for ecophenotypic variants as clinically and neurobiologically distinct subtypes. Am. J. Psychiatr. 170, 1114–1133. https://doi:10.1176/appi.ajp.2013.12070957.

Teicher, M.H., Samson, J.A., Anderson, C.M., Ohashi, K., 2016. The effects of childhood maltreatment on brain structure, function and connectivity. Nat. Rev. Neurosci. 17, 652–666. https://doi:10.1038/nrn.2016.111.

Vaillancourt, K., Ernst, C., Mash, D., Turecki, G., 2017. DNA methylation dynamics and cocaine in the brain: progress and prospects. Genes 8 (5), 138. https://doi:10.3390/genes8050138.

Ventura, R., Cabib, S., Puglisi-Allegra, S., 2002. Genetic susceptibility of mesocortical dopamine to stress determines liability to inhibition of mesoaccumbens dopamine and to behavioral 'despair' in a mouse model of depression. Neuroscience 115, 999–1007. https://doi:10.1016/s0306-4522(02)00581-x.

Walton, E., Hass, J., Liu, J., Roffman, J.L., Bernardoni, F., Roessner, V., et al., 2016. Correspondence of DNA methylation between blood and brain tissue and its application to schizophrenia research. Schizophr. Bull. 42, 406–414. https://doi:10.1093/schbul/sbv074.

Wang, H.R., Woo, Y.S., Ahn, H.S., Ahn, I.M., Kim, H.J., Bahk, W.-M., 2015. The validity of the mood disorder Questionnaire for screening bipolar disorder: a meta-analysis. Depress. Anxiety 32, 527–538. https://doi:10.1002/da.22374.

Wang, H.-T., Huang, F.-L., Hu, Z.-L., Zhang, W.-J., Qiao, X.-Q., Huang, et al., 2017. Early-life social isolation-induced depressive-like behavior in rats results in microglial activation and neuronal histone methylation that are mitigated by minocycline. Neurotox. Res. 31, 505–520. https://doi:10.1007/s12640-016-9696-3.

Webb, L.M., Phillips, K.E., Ho, M.C., Veldic, M., Blacker, C.J., 2020. The relationship between DNA methylation and antidepressant medications: a systematic review. Int. J. Mol. Sci. 21, 826.

Wossidlo, M., Nakamura, T., Lepikhov, K., Marques, C.J., Zakhartchenko, V., Boiani, M., et al., 2011. 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat. Commun. 2, 241. https://doi:10.1038/ncomms1240.

Zhang, T.-Y., Hellstrom, I.C., Bagot, R.C., Wen, X., Diorio, J., Meaney, M.J., 2010. Maternal care and DNA methylation of a glutamic acid decarboxylase 1 promoter in rat hippocampus. J. Neurosci. 30, 13130–13137. https://doi:10.1523/JNEUROSCI.1039-10.2010.

Zhu, Y., Strachan, E., Fowler, E., Bacus, T., Roy-Byrne, P., Zhao, J., 2019. Genome-wide profiling of DNA methylome and transcriptome in peripheral blood monocytes for major depression: a Monozygotic Discordant Twin Study. Transl. Psychiatry 9, 215. https://doi:10.1038/s41398-019-0550-2.