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REVIEW ARTICLE

Intergenerational Transmission of DNA Methylation Signatures Associated with Early Life Stress

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Abstract: Early life stress in humans (*i.e.* maltreatment, violence exposure, loss of a loved one) and in rodents (*i.e.* disrupted attachment or nesting, electric shock, restraint, predator odor) occurs during critical steps of neural circuit formation. ELS in humans is associated with increased risk for developmental psychopathology, including anxious and depressive phenotypes. The biological mechanisms underlying these potentially persistent maladaptive changes involve long-term epigenetic modifications, which have been suggested to be potentially transmissible to subsequent generations. DNA methylation is an epigenetic mechanism that modifies gene expression patterns in response to environmental challenges and influences mutation rates. It remains to be seen whether a functionally relevant fraction of DNA methylation marks can escape genome-wide erasures that occur in primordial germ cells and after fertilization within the zygote. Early life-stress-triggered changes in epigenetic mediated transmission of acquired behavioral traits among humans have been assessed mainly by targeting genes involved in the hypothalamic-pituitary-adrenal (HPA) axis, such as NR3C1 and FKBP5. Recently, researchers examining epigenetic transmission have begun to apply genome-wide approaches. In humans, reduced representation bisulfite sequencing (RRBS) was performed on peripheral samples that were obtained from individuals who were prenatally exposed to the "Dutch Hunger Winter", resulting in two Differentially Methylated Regions (DMRs) in INSR and CPTIA genes that were functionally, biologically and technically validated, and significantly associated with birth weights and LDL cholesterol levels in offspring. In rodents, non-genomic intergenerational transmission of anxiety which was associated with differentially methylated enhancers that were putatively involved in lipid signaling and synaptic/neurotransmission in hippocampal granule cells, was discovered also using RRBS. Finally, transgenerational transmission of altered behaviors was associated with sperm-derived microRNAs produced by ELS male mice. The field of epigenetic transmission is just beginning to enter the epigenomic era by using genome-wide analyses. Such approaches remain of strong interest to human studies, first in order to help to assess the relevance of the previous targeted studies, and second to discover new important epigenetic modifications of potential clinical importance. New discoveries may help to assess how transmittable the negative impact of stress may be to offspring. The latter may open doors for future treatments and resilience-promoting interventions, as well as new approaches to treat the effects of childhood trauma before the onset of psychiatric disorder.

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1. INTRODUCTION

1.1. DNA Methylation

The existence of methylated cytosine was first reported in 1925, having been observed in a non-canonical nucleic acid that had been isolated from the tuberculous bacillum. It is from this "tuberculinic acid" that DNA itself had been discovered [1]. Years passed before that unusual fifth DNA base called "5-methyl-cytosine" (5mC), in which an additional methyl group is attached to the 5th atom of the pyrimidine ring of the cytosine, was found to be ubiquitously present in the DNA across numerous different species from bacteria to humans. A notable exception was encountered in the nematode *Caenorhabditis elegans* that did not contain 5mC but instead carried adenine N6-methylation (6mA) [2]. 5mC was considered as absent from yeast genomes for years, before detection of 5mC across yeast strains DNA using gas chromatography combined with mass spectrometry [3]. Another work later highlighted that the method used was unable to distinguish DNA- from RNA-derived 5-mC, and reestablished the dogma that 5mC is absent from yeast DNA [4]. In *Drosophila*, one of the most studied organisms in the field of developmental genetics, 5mC was also considered absent prior to the discovery of extremely low levels of 5mC by virtue of new technology, and prior to the subsequent discovery of higher levels of dynamic 6mA implicated in proc-

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esses of early development [5]. In the DNA of differentiated mammalian cells, 5mC has been localized within 5' cytosine phosphate guanine 3' (CpG) sequences. The reasons for this specific localization may be due to the palindromic property of that dyad, allowing methylation in both DNA strands at the same site. In mammalian cells, 5mC represents 3 to 8% of total cytosine, and 70 to 80% of CpG cytosine are methylated on both strands in human somatic cells [6].

Cytosine methylation in DNA appears to exert essential regulatory functions within mammalian genomes. One of the first pieces of evidence for the regulatory function of DNA methylation on gene expression came from experiments involving the electroporation of 5-methyl deoxycytidine-triphosphate in Chinese hamster ovaries. The latter resulted in the inactivation of several genes that code for pertinent enzymes. In these first experiments, the effects were reversed by treating clones with 5-azacytidine, a demethylating agent. That work provided some of the first experimental evidence showing "a direct relationship between DNA methylation and gene silencing" [7]. But it remained unclear whether DNA methylation within a promoter region caused gene repression, or whether DNA methylation was a consequence of gene repression, that contributed to the stabilization of the repressed state of the gene by acting on the chromatin [8]. Some researchers consider DNA methylation as a 'lock' to maintain and/or reinforce a previously silenced state of a given gene. In both instances, methylation-dependent control of gene expression involves the repressor methyl CpG binding protein 2 (MeCP2). The latter binds specifically to DNA methylated CpGs with a resolution of 12 base-pairs *via* its methyl-CpG binding domain (MBD), whereas its N-terminal region may induce the methylation of lysine 9 of histone H3 [9]. MeCP2 contains several additional chromatin specific binding domains [10]. Thus, MeCP2 would bind to methylated DNA and induce the modification of histones, which in turn, results in changes in the three-dimensional structure of the DNA molecule. These changes determine the accessibility of the DNA molecule to the transcriptional machinery in order to start transcription. Additional studies revealed that DNA methylated CpGs bind not only to MeCP2 but also to other specific proteins: namely, MBD1, MBD2 and MBD3 that in turn deacetylate surrounding histones [8]. The actual consensus remains that methylation of CpG in CG rich promoters represses genes indirectly *via* mechanisms involving histone deacetylation and consecutive chromatin compaction that prevents transcription.

The methyl-group found in 5mC is thought to have originated in the folate cycle, and then to have been incorporated into the cytosine molecule within the DNA by the DNA cytosine methyltransferase (DNMT) enzymes *via* the methionine cycle. This process is a reaction involving the methyl donor molecule S-adenosyl-L-methionine (SAM) (Fig. **1**). Six different DNMTS enzymes have been character-

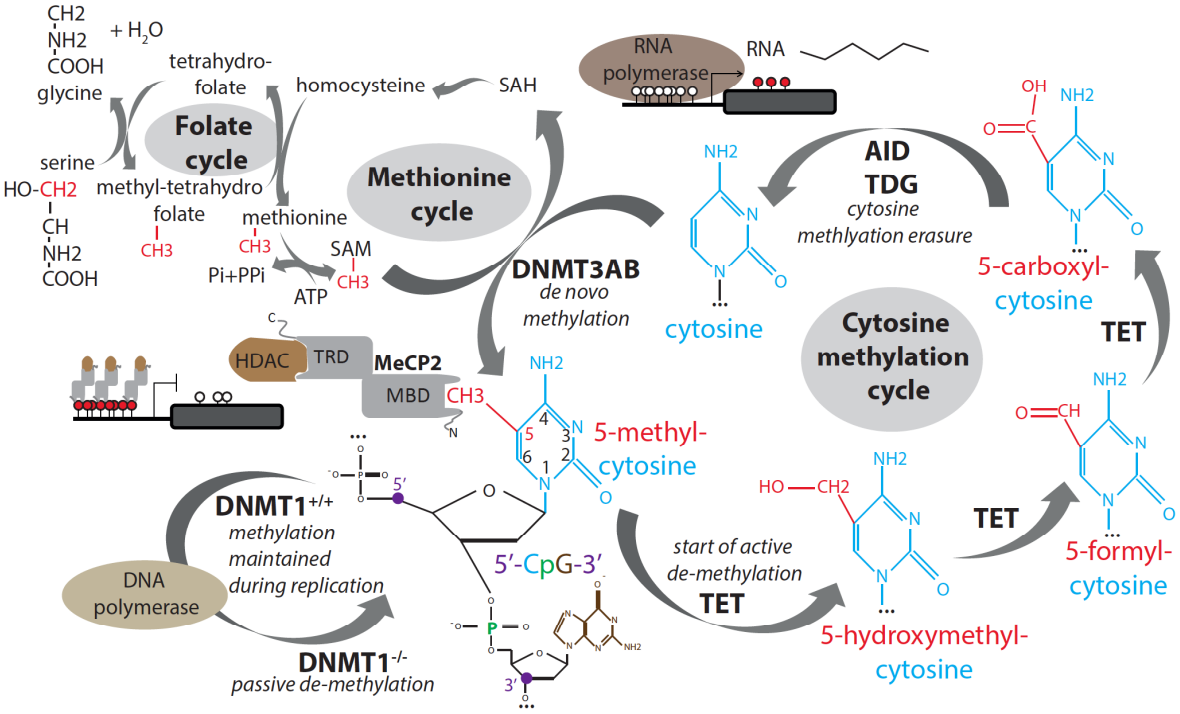

Fig. (1). Biochemical cycles and DNA modifying enzymes involved in methylation and de-methylation of mammalian cytosine at the 5th atomic position and its consequences on gene expression. The methyl group is shown in red and is transferred from S-adenosyl-L-methionine (SAM) donor to cytosine by *de novo* DNA methyltransferases enzymes (DNMT3AB). Active demethylation of methylated cytosine involved ten-eleven-translocation proteins (TET), activation-induced deaminase (AID) and thymine DNA Glycosylase (TDG) enzymes. Passive dilution of methylation may occur by replication in the absence of DNMT1 activity. Highly methylated CG rich promoter associated with de-methylated gene body is usually silenced. The methyl-CpG binding domain (MBD) of MeCP2 binds methylated CpG, and the transcriptional repressor domain (TRD) of MeCP2 recruits indirectly histone deacetylases (HDAC). HDAC activity triggers chromatin compaction and transcriptional silencing. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

ized so far: DNMT1, DNMT2, DNMT3A, DNMT3A2, DNMT3B, DNMT3L, that exert different functions [11]. Inactivation of Dnmts in mice is lethal and associated with hypo-methylation of DNA and with increased DNA damage and instability. Dnmt1 appeared to be an essential contributor to the maintenance of CpG methylation through the DNA replication occurring during cell division, and to possess the ability to methylate a hemi-methylated CpG site, *i.e.* a CpG site in which the methylated cytosine is present only in one of the two DNA strands. DNMT2, the most conserved DNMT, surprisingly failed to show any evidence of an essential molecular function with respect to the DNA molecule, until the discovery that this particular Dnmt enzyme was able to methylate transfer-RNA [12]. In this respect, DNMT2 may become known as a key player in the emerging field of epi-transcriptomics. DNMT3A is responsible for germ-cell maintenance, and is involved in the establishment of imprinting, *i.e.* gene expression under the control of only one of two parental alleles within a given cell. DNMT3A2 is a distinct isoform of DNMT3A found in the male germ-cell and probably involved in *de novo* methylation after the first methylation erasure as discussed below. *Dnmt3B* is essential. Mutations in the corresponding human gene homologue are responsible for the disorder known as Immunodeficiency, Centromere instability and Facial anomalies syndrome (ICF). Absence of *Dnmt3B* in cells may result in partial loss of DNA methylation and either premature senescence or spontaneous cell immortalization [13]. *Dnmt3L* is expressed specifically in germ-cells and is essential for establishing maternal imprinting.

Mammalian cells possess two alleles, one from each parent. In some genes, called imprinted genes, one allele is silenced by methylation depending on its parental origin in a process called imprinting. In germ lines, a single copy of the gene is present, and a demethylation process occurs. In fact, two developmental periods have been identified in which global demethylation is followed by global re-methylation of DNA.

2. DNA METHYLATION ERASURE AND REPROGRAMMING

During the passage from one generation to the next, the great majority of the DNA methyl marks are erased. This includes the "physiological" methyl marks that are established in each generation during development and in adulthood and the "accidental" methyl marks induced by environmental factors. The DNA methylation erasures or "reprogramming" occurs in two major waves affecting two different cell types. The first major wave of de-methylation begins just after the fertilization of the oocyte by a spermatozoon, resulting in a zygote that contains the two pro-nuclei of biparental origin that are physically separated from one another as two different nuclear entities within the same cytoplasm. The second major wave of reprogramming occurs before meiosis in primordial germ cells (PGC) of the developing embryo, at the time PGCs in G2 phase enter the developing and differentiating gonad, and corresponding in the mouse to embryonic days E11.5 to E12.5 [14].

With regards to the first reprogramming wave, asymmetric reprogramming occurs in both zygotic pro-nuclei that will then fuse together to produce a new, globally un-methylated, diploid somatic genome associated with cell totipotency. By contrast, *de novo* re-establishment of DNA methylation is associated with the loss of totipotency after the implantation of the blastocyst in the endometrium, for review see Cantone & Fischer, 2013 [15]. During this first wave of reprogramming within the zygote, the sperm derived pro-nucleus has been estimated to be twice as methylated as the pro-nucleus of the oocyte [15]. In the paternal pro-nucleus that is located within the zygote, DNA methylation is actively erased within 6 to 8 hours [16], and protamines are replaced with histones of maternal origin [17]. Active de-methylation of the paternally transmitted pro-nucleus is supported by the observation of high expression of Tet Methylcytosine Dioxygenase 3 (TET3) in the zygote and accumulation of oxidative products of 5mC, *i.e.* 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5Fc) and 5-carboxycytosine (5caC), detected specifically in paternal sister chromatids during the first zygotic metaphase [18, 19]. These oxidative products are implicated in active de-methylation of the methylated cytosine mediated by members of the ten-eleven-translocation proteins (TET), (Fig. **1**) [20]. For the remaining portion of the paternally transmitted pro-nucleus, some histones are retained in the sperm and inherited *via* the zygote at particular methylated DNA sequences resisting TET3 oxidation, the latter, which are probably related to paternal imprinting [15, 21].

The maternally derived pro-nucleus differs from the paternally derived pro-nucleus in several aspects: it is arrested in metaphase II; the DNA is less compacted and twofold less methylated, and is surrounded exclusively by histones. The latter differs from the paternally transmitted pro-nucleus which is almost exclusively surrounded by protamines. It is though that DPPA3 (also named PGC7) protects the maternal genome from Tet3-mediated oxidation of 5mC by its binding to H3K9me2 histone initially present almost exclusively in the maternal pro-nucleus; whereas, Tet3 is enriched specifically is the male pro-nucleus. Thus, an active demethylation process by TET3 is considered to be absent in the maternally transmitted pro-nucleus [22]. Passive demethylation is thought to occur in the maternally derived pro-nucleus. But, in replicated maternal pro-nucleus, Dnmt1 activity remains detectable and was required for the maintenance of maternal methylated imprinted regions [23]. It remains a mystery, how exactly the passive de-methylation of marks occurs outside imprinted loci during the replication of the maternal pro-nuclei despite the presence of Dnmt1 activity, and why that mechanism affects differently the imprinted loci in contrast to loci that are subjected to erasure [15].

The second wave of reprogramming occurs in primordial germ cells (PGCs) [14]. Sequential demethylation in PGCs is observed with a first stage of initial two-fold global decrease of DNA methylation which is associated with PGC migration from epiblast to gonads that remains independent from the TET enzymes [24]. The latter is followed by a second locus specific TET-dependent stage of active demethylation observable between E10 and E14 and involving Tet1 dependent oxidation of 5mC to 5hmC, resulting in demethylation then activation of genes involved in meiosis which is part of the second major wave of reprogramming specifically affecting PGCs [14]. It may well be that the first PGCs

originated from the posterior epiblast as 40 pioneering germline cells that begin to migrate at E7 from epiblast to the hindgut endoderm, where they constitute the basis for the future gonads of the developing embryo [15]. Migration of PGCs continues up to embryonic days E11.5 to E12.5 after triggered sexual differentiation to either a testis or an ovary beginning at E10. During stage I and despite global demethylation on a genomic scale, both imprinted control regions and meiotic genes remain methylated by Dnmt1 activity in PGCs [25].

As mentioned before, the second major wave of reprogramming is TET-dependent and involved active demethylation of 5mC to 5hmC between E10 and E14. 5hmC conversion to cytosine is then thoughts to depend on base excision repair (BER) mechanisms involving cytidine deaminases (AID) [26]. After the second wave, female PGCs are less methylated than male ones [26]. The imprinted loci are considered to be subjected to de-methylation during the second major wave of Tet-mediated reprogramming in PGC at E14, but some loci resists erasure and reprogramming, notably retrotransposon and intracisternal A particle (IAP) genetic elements [27]. It has to be mentioned that DNA methylation interacts with histone modifications during reprogramming. Briefly, methylation of lysine 27 of histone 3 (H3K27) represses developmental genes during both waves of DNA methylation erasures [28]. Secondly, as shown by the results from the "Grandiose Project," regions of DNA detected as having been differentially methylated during reprogramming showed more than 10-fold enrichment in methylation on lysine 9 of histone 3 (H3K9) [29]; whereas, methylation of the DNA sequences of silenced transposons not resisting erasure are usually surrounded by H3K9 [28].

3. INTERGENERATIONAL TRANSMISSION OF DNA METHYLATION

The intergenerational transmission of epigenetic modifications, *i.e.* of changes in gene expression without changes in DNA sequences, is defined as observable epigenetic changes that are transmitted from one generation to the next, or from parent's generation, named the zero filial generation (F0), to the offspring's generation, named the first filial generation (F1) [30]. Intergenerational transmission is distinguished from transgenerational transmission, in that the latter implies continuing transmission across generations, from F1 offspring to their respective offspring or second filial generation (F2), without exposure to the initial environmental stressor. Note that in the case of maternal to offspring transmission with *in utero* exposure to a stressor; F1 embryos growing into F0 pregnant mothers already contain germ cell precursors that will generate the F2 individuals. Thus, F0, F1, F2 cells are exposed to the initial stressor. Transgenerational inheritance in these cases has therefore been strictly considered to begin only from the third filial generation (F3) onwards.

Several studies reported epigenetic transmission across generations suggesting incomplete erasure of methylation marks within the germ-line. First, an imprinted murine transgene called "DM2" was microinjected into pronuclei of fertilized oocytes in order to generate a transgenic mouse line. The DM2 transgene contained a globulin promoter (*i.e.* a sequence required to initiate transcription), a Shine-Dalgarno and Kozak sequence (*i.e.* respectively prokaryotic and eukaryotic ribosomal recognition sequences required for the RNA to be translated into protein), a *LacZ* reporter (*i.e.* a gene used as reporter, coding for β-galactosidase enzyme, whose activity may be seen by the production of an insoluble blue compound upon hydrolysis of X-GAL) with a polyadenylation signal (*i.e.* a sequence required to terminate the transcription) as well as an enhancer (*i.e.* a sequence that is able to increase the transcription). In the filiation produced by crossing the transgenic line with the opposite wild-type sex, the presence, activity and methylation of the transgene were measured. In the filiation produced by crossing the transgenic line with the opposite wild-type sex, the presence, the activity and the methylation of the transgene were measured. Results followed up to four consecutive generations showed that the transgene was always present; but its activity was silenced by DNA methylation in some but not all offspring following female transmission. The imprint was not completely erased in the germ-line and produced an unusual, non-Mendelian pattern of inheritance [31]. The rules governing this mode of transmission remain unknown.

Second, the "*agouti*" locus presented epigenetic inheritability [32]. The specific locus called *agouti* (A) in mice encodes a paracrine signaling protein causing melanocytes to switch from the synthesis of a black pigment to a yellow one (phaeomelanin). Isogenic viable yellow mice carrying agouti (A^{vy}) present in fact coats that vary gradually between full yellow with high expression of A^{vy}, to brown called pseudo-agouti color associated with low expression of A^{vy}. These easily viewable phenotypical variations occur without genetic changes, but with variation in the transcriptional activity of an intra-cisternal A particle (IAP) located upstream of the agouti locus where the transcription starts. A^{vy} mice were crossed with recessive agouti null mice $^{a/a}$ producing heterozygote offspring $A^{vy/a}$, further classified based on the color of the coats. Results showed methylation of the AIP in $A^{vy/a}$ mice of pseudoagouti color, absence of methylation of the AIP in $A^{vy/a}$ mice of yellow color. When mother has silenced A^{vy} allele with agouti phenotype, more offspring present agouti phenotype with silenced A^{vy} allele. This showed that the incomplete erasure of the silenced A^{vy} allele passed through the female germ line, with as a consequence, inheritance of the epigenetic modification determining the phenotypical trait of the coat color.

Third, in outbred mice prenatally exposed to vinclozolin, transgenerational transmission of adult onset diseases associated with modifications in the mouse sperm epigenome across generations has been reported [33]. Analysis performed on sperm showed also a transgenerational impact of prenatal exposures to vinclozolin or methoxychlor on methylation levels in some imprinted gene CpGs in F1 offspring with gradual reestablishment to normal levels in F2 and F3 offspring [34, 35]. Finally, in humans, the inheritance from the mother to one of its three sons of a cancer-associated germ line dysmethylation was reported in the *MLH1* promoter [36]. Despite these observations, transmission of methylated DNA marks from parent to offspring germ-line cells in humans remains a conflictual area [37]. The mechanisms underlying this apparent resistance to erasure-reprogramming of some epigenetic marks are unknown.

Analysis of individual CpGs has shown that some are resistant and some are not to erasure-reprogramming, suggesting a specific protection of some CpGs that might be due to the chromatin environment [34, 35]. Other types of epigenetic modifications, such as histone acetylation, may also contribute to the inheritance of particular phenotypes.

The results of studies on epigenetic transmission showed that environmentally induced epigenetic effects are frequently not transmitted, or are only occasionally transmitted then lost, and, at best, only rarely stably transmitted to the subsequent lineage [30]. Direct evidence for the heritability of stochastic epigenetic variations has been reported that might explain developmental plasticity at certain loci [38]. As mentioned previously, epi-mutation such as an abnormal methylation of a DNA mark have to resist DNA methylation erasure waves in order to be transmitted to subsequent generations. But alternatively, *cis*- and *trans*-acting factors may reestablish an epi-mutation after its erasure. In addition, CpG sites can be acquired or lost by genetic mutation. Therefore, methylation of DNA interacts with mutational rates in a sequence context dependent manner [39]. Mutations in DNA are frequent and are subject to environmental influences. The DNA repair machinery corrects mutations. This machinery, for example, is able to replace a wrong uracil by a correct thymine after hydrolytic spontaneous deamination of cytosine. However, the deamination of methylated cytosine actually produces thymine rather than cytosine. Since thymine is not corrected, transmissible genetic mutations are produced. The mutation rate at CpG sites of 5mC involving thymine has been estimated to be 10 to 50 folds higher than other mutations. The so-called CpG islands (CGI) are globally less subject to mutation than isolated CpGs. Why CpG sites are more likely to be methylated when isolated within the surrounding sequence, as compared to CpG clustered together, is not known.

4. EARLY LIFE STRESS IN ANIMAL MODELS

In rodents, a variety of early life stress (ELS) models have been developed, see review by Schmidt *et al.* [40]. The first originally developed model involved rats [41] that were subjected to a daily manipulation called "early handling" (EH). Handled pups were compared with non-handled (NH) pups and with an animal facility rearing (AFR) group. EH involved daily physical manipulation of the litter and separation of the pups from the mother for short periods of time. NH litters were not exposed to any physical human disturbance; whereas, AFR litters were exposed to animal facility rearing as-usual. A second type of paradigm was derived from EH mice, either by removing the pups from the litter in early deprivation (ED), or by removing the mother from the litter in the so-called maternal separation (MS) model. The MS experimental model may be performed either within 24 hours and involving only a single occurrence, or repetitively, most often for a period of 1 to 8 hours. Variations of this MS model involved additional manipulation of the separated mother as for example in the instance of "unpredictable maternal separation combined with unpredictable maternal stress" (MSUS), separating mice mothers from their pups at "any time during the dark cycle" daily, and then either placing the mothers in a restraint or in a forced-swim task [42, 43]. Finally, modification of cage contents such as by providing scant nesting material was also used to induce ELS [44]. By contrast, other techniques of disrupting the caregiving environment included the following: enrichment of the nesting material (EE) by placing additional objects in the cage; handling and changing the nesting materials, which perturbs nesting; and forcing animals to share a communal nest (CN) in which three females are placed in the cage to nest until weaning. These disrupted caregiving environment conditions have all been compared to standard laboratory conditions. It is important here to clarify that CN is considered to be ethologically a better reflection of a natural nest as compared with a typical laboratory nest, and thus results in a positive impact on the mice. CN improved postpartum maternal care, reduced anxiety-like behavior of offspring in adulthood when confronted with a novel environment, and reduced aggression of adult female offspring when confronted with a male intruder [45]. Of note, female BALB/c mice mated with males that were grown in CN exhibited increased levels of pup nursing even in the absence of the father during the postpartum period [46]. This suggests that paternal social experience influences maternal investment in offspring in mice. Pharmacologic interventions have also been used to mimic ELS, such as for example injecting dexamethasone, a synthetic substance that mimic the action of stress-evoked glucocorticoids. Finally, non-interventional models of ELS observed variations in maternal behavior towards pups. Results showed that the genetic backgrounds may interfere with ELS mice models. Moreover, mice are more resistant to MS as compared with rats. It is probably for these reasons, that the MSUS model was developed in mice [47]. The addition of unpredictable maternal stress to the MS paradigm in MSUS model aggravates the behavioral responses transmitted to male offspring according to open field, open field emergence test and elevated plus maze test [48]. Results of the experiments showed notably reduced latency to enter the novel open field observed in F1 mice derived from MSUS paradigm, which was not the case in F1 mice derived from unpredictable MSU.

5. EARLY LIFE STRESS AND RISK FOR PSYCHIATRIC OUTCOMES

ELS in humans is a very broad concept that generally refers to excessive or "toxic" stress to which the organism is exposed during sensitive periods of early brain development. The main damage results from an unpredictable environment in which the vulnerable, young, developing organism integrity or survival are threatened [49-51]. It may also be due to difficulties in the attachment relationship upon which the young organism depends. According to the diathesis - stress perspective, the brain may be damaged by excessive exposure to stress to which it is particularly sensitive during its developmental period [52]. The greatest impact of ELS is thought to occur during the following formative periods: the prenatal, the early postnatal and the early adolescent periods [40]. Stress occurring before conception (pre-conception phase) may also impact cortical circuits of future offspring *via* DNA methylation according to several recent studies that are discussed below. Nevertheless, the pre-conception stress is not considered to be truly "ELS" since it has occurred prior to the existence of the living offspring.

In the majority of the studies reviewed, the assessment of ELS in human samples was achieved *via* retrospective self-report questionnaires. These measures generate quantifiable scores for a variety of types of early life stressors such as physical, sexual and emotional abuse, physical and emotional neglect, separation and loss. These questionnaires are the following: The Child Trauma Questionnaire (CTQ) [49] and its short form (the CTQ-SF), the Childhood Experience of Care and Abuse (CECA) questionnaire [50], the Adverse Childhood Experiences (ACE) and the Maltreatment and Abuse Chronology of Exposure (MACE) [53]. In humans, childhood maltreatment is recognized as risk factor for a large number of psychiatric disorders [52].

The "Welsh ACE study" (http://www.cph.org.uk/) tested adverse childhood experiences *via* self-report questionnaires that were completed by 9'507 medically evaluated individuals. Those exposed to four or more types of adverse childhood experiences (ACEs) showed a 4- to 12-fold increase in their risk for alcoholism, drug abuse, depression, and suicide attempts, as compared to those who had experienced no ACEs [51]. In a cross-sectional survey based on 8'580 adults of aged between 16 and 74 from the second British National Survey of Psychiatric Morbidity, childhood sexual abuse increased the risk by 2.9 odd ratio (1.3-6.4) for psychosis during adulthood [54].

The connection between exposure to childhood maltreatment and the delayed pathogenesis of psychiatric disorder during adulthood is currently under study [55]. One of the largest register-based population cohort studies to date [56], recently published findings that included all individuals born in Denmark from 1980 to 1998, this after excluding both émigrés and individuals who either had died or were diagnosed as having bipolar disorder prior to 15 years of age. In this latter study of 980'554 participants, despite the observation that single exposure to most of the adversities probed prior to age 15 increased the risk for bipolar disorder after age 15, the presence of any mental disorder in the parents was the strongest associated risk factor for bipolarity among the offspring, with a hazard ratio of 3.53 (2.73-4.53). The authors concluded that bipolar disorder has a strong genetic loading, therefore challenging the notion that early-life events are the primary determinant of its onset [56].

6. TARGETED EPIGENETIC STUDIES INVOLVING THE HPA-AXIS: THE CASE OF HIPPOCAMPAL NR3C1

Mammals have developed various mechanisms to adjust their behavior in response to threatening conditions by activating physiological responses that permit them to fight, flee or freeze. The hypothalamic-pituitary-adrenal (HPA) axis has been found to respond to stressors directly as well as *via* feedback-mechanisms. The adrenal glands are responsible for the secretion of glucocorticoid stress hormones. These hormones stimulate notably gluconeogenesis in order to provide energy to sustain the fight or flight in the presence of a perceived threat. When stress occurs during brain development at toxic levels and/or repeatedly over time, without a sufficient period of time for recovery, the HPA-axis may chronically become dysregulated [57]. As DNA methylation was suggested to have the ability to stabilize genomic expression across the lifespan in response to environmental cues, it was hypothesized as a putative biological support for long-lasting stress-related dysregulation that affect the brain and modifying behaviors.

The first discovery along these lines was that natural variations in rat-dam-maternal behavior towards their respective pups were associated with changes in DNA methylation of the glucocorticoid receptor gene (*Nr3c1*) promoter regions within the hippocampus, beginning during early life and persisting up through adulthood in the offspring. Pups born to low-licking and grooming mothers showed increased methylation levels in the exon 1_7 of the *Nr3c1* gene, coding the stress hormone receptor, more specifically at two CpG sites numerated sites 16 and 17 located at both ends of its 5'-$G(CG)_{16}GGGG(CG)_{17}$-3' binding site for the neural transcription factor NGFI-A. Increased DNA methylation was associated with decreased histone acetylation, as a marker of active chromatin, as well as with decreased production of NR3C1 proteins. Cross-fostering the pups born to low- licking and grooming mothers with high-licking and grooming adoptive mothers resulted in a methylation status at the 5'-GCGGGGGCG-3' binding site that was indistinguishable from that of pups born to and reared by high-licking and grooming dams. These results demonstrated that the maternal rearing behavior towards the pups was responsible for changing the methylation status in NGFI-A binding site of the glucocorticoid receptor. Intra-cerebroventricular infusion of 100 ng/ml of the histone deacetylase inhibitor trichostatin A to the adult offspring decreased the degree of cytosine methylation of both sites 16 and 17 in the NGFI-A binding region [58].

More recently, both F1 and F2 generations derived from C57BL/6J mother mice that were exposed to ELS by using the MSUS method, presented altered behavior that was associated with decreased promoter methylation and increased expression of hippocampus NR3C1. Both behavioral and biological alterations were, however, reversible by environmental enrichment [42]. This work demonstrates both intergenerational and transgenerational transmission of ELS-induced alterations of behavior and of DNA methylation status and additionally highlights the reversibility of these alterations through modification of the environment in mice.

ELS induced alterations in NR3C1 was also reported in other organisms. In *Rhesus macaque*, DNA methylation and gene expression were analyzed by comparing two groups of animals, grown either in the presence or in absence of their mothers, and examining effects in two different tissues: namely, the prefrontal cortex and peripheral T cells. Results showed that the promoter of the macaque *A2D681* gene, homologue to *Nr3c1*, was more methylated in both tissues in animals that were raised without mothers [59]. In humans, analyses performed on postmortem hippocampus showed increased levels of CpG methylation in *Nr3c1* promoter and decreased levels of *Nr3c1* transcripts in victims of childhood abuses [60]. With respect to *in vitro* studies, inserts containing full length *Nr3c1* promoter or the promoter deleted for the NGFI-A binding site were cloned into a reporter plasmid and were either methylated in their CpG or not by in-vitro patch methylation. Luminescence was monitored in presence or absence of the NGFI-A protein. Results showed decreased

luminescence when patch-methylation was performed and increased luminescence in presence of NGFI-A protein, compatible with a negative impact of methylation and a positive impact of NGFI-A on *Nr3c1* promoter activity [60]. Thus, *in vitro* studies suggest that the increased levels of CpG methylation in *Nr3c1* promoter observed in the hippocampus of human victims of childhood abuse might explain the decreased level of *Nr3c1* transcripts observed in this same brain region. Recently, DNA methylation differences were reported between grey and white matters and in genes that were previously associated with Alzheimer, Parkinson, Huntington, and Multiple Amyotrophic lateral sclerosis diseases [61].

7. PERINATAL STRESS AND BRAIN PROGRAMMING

Perinatal stress coincides in time with neurogenesis, migration of progenitors and establishment of circuits and synapses. Epigenetic changes in embryonic neural cells or their precursors may thus putatively interfere with neurogenesis, neuronal differentiation, and synaptic wiring patterns. Several different reviews summarized evidence indicating that the epigenome is supporting long-lasting effects of stress occurring during pregnancy on the development of the brain and the subsequent behavior that is recorded in the offspring [62, 63]. These reviews additionally highlight that exposure to stress in-utero may alter neuronal circuits in specific brain regions rather than affecting the entire brain in a uniform manner, albeit sometimes with conflicting results. For example, the review of Bock *et al.* reported three different animal studies in rats that involved perinatal stress and that was associated with the observation of decreased dendritic length and decreased dendritic tree complexity in the hippocampus area called "Cornu Ammonis 3" (CA3) of the offspring - this being a brain area implicated in the rapid acquisition of novel information or short-term memory. In human models, one retrospective and two prospective studies revealed associations between stress during pregnancy as reported by the mothers in addition to mental development index scores that were measured in the offspring. Importantly, differences were reported depending on the interaction of the sex and environmental epigenetic programming in terms of vulnerability and resilience that may well be related to hormone-mediated mechanisms.

8. TARGETED EPIGENETIC STUDIES AND BRAIN ACTIVITIES DURING MOTHER-CHILD INTERACTIONS: CONVERGENCE TO THE MEDIAL PREFRONTAL CORTEX

In a sample of mothers that included women with interpersonal violence-related posttraumatic stress disorder (IPV-PTSD), human brain activity and possible association with DNA methylation changes in genes involved in the stress response were studied during interactions occurring between mothers and their children. A behavioral protocol was settled involving both free-play and mother-child separation conditions. Additionally, maternal neuronal activity and DNA methylation were measured respectively using fMRI imaging and bisulfite-pyrosequencing from saliva extracted DNA, on several targets, *NR3C1*, Brain-Derived Neurotrophic Factor (*BDNF*) and ionotropic receptor of serotonin (*HTR3A*).

The first study targeting *NR3C1* showed that parenting stress and maternal PTSD were negatively correlated with mean methylation levels of peripheral *NR3C1*. Moreover maternal neural activity on fMRI within the medial prefrontal cortex (mPFC) in response to video-stimuli of mother-child separation *versus* play was positively correlated with *NR3C1* methylation [64].

The second gene explored was *BDNF*. This gene was chosen because elevated levels of circulating glucocorticoids are thought to interfere with BDNF signaling [65]. Moreover, *BDNF* promoter methylation levels compared in post-mortem peripheral tissues and brain regions extracted from the same individuals were significantly correlated [66]. This result suggested that in case of *BDNF* methylation, measurements performed in peripheral tissues may be a good proxy for the brain levels in term of inter-individuals differences. The question of whether observed DNA methylation changes in peripheral tissues may reflect differences in brain regions is almost never tested. Note that both tissues tested were postmortem quadriceps and mPFC but we do not know if the DNA extracted from saliva may also be a valuable proxy. Results showed that maternal *BDNF* methylation in saliva was positively correlated with higher levels of maternal anxiety and greater childhood exposure to domestic violence, and that brain activity measured by fMRI was positively correlated with *BDNF* methylation in both the anterior cingulate (ACC) and the ventromedial prefrontal cortex (vmPFC) [67].

A third report using similar methodology targeted the *HTR3A* gene. Genetic variation in human *HTR3A* gene may impact emotional brain and may contribute to risk for major depression in the context of a history of ELS [68]. *HTR3A* methylation at the so-called "CpG2_III" site was linked to decreased medial prefrontal cortical activity in response to menacing relational stimuli [69]. This site was of particular interest because of its localization just before RS1062613 polymorphism within a CTCF binding site. Childhood physical abuse was significantly impacting the history of suicide, mood episodes and hospitalization mediating the methylation level at that site in psychiatric cohorts [70].

To conclude, this new experimental approach based on measurements of methylation levels in stress related genes and functional assessment of brain activities during experimental situations produced interesting new insights into functional brain imaging and epigenetics. Note that IPV-PTSD mothers and their children showed blunted salivary cortisol reactivity to laboratory stressor, compatible with epigenetic dysregulation impacting genes involved in the stress axis in both mothers and toddlers [71]. The global findings of three different targeted studies converged to the mPFC area, in which neuronal activities during mother-children stressful situations tend to correlate with peripheral marker methylation in stress related genes. Interestingly, the mPFC area of the brain may generate emotions and is connected with the limbic system [72]. Further ongoing development in fMRI technology using detectable sensors would allow to monitor neurotransmitter kinetics *in vivo*. This was reported for the serotonin sensor called "2G9C6", whose dissociation from the serotonin molecule allowed for quantitative measurement of the degree of serotonin transport [73].

In animal studies, the application of such techniques in association with DNA methylation measurements in brain regions activated by neurotransmitter may further help to assess the role of DNA methylation in brain functions.

9. MATERNAL STRESS DURING PREGNANCY MAY EPIGENETICALLY CONTRIBUTE TO THE INTERGENERATIONAL TRANSMISSION OF ANXIETY

Experiments performed using mouse-models have highlighted the fact that changing the maternal environment during pregnancy may modify a behavioral trait in the offspring. The processes involve epigenetic modifications that affect enhancer sequences detected in specific brain regions controlling notably sphingolipids and lipid signaling in neuron membranes [74]. More specifically, two intergenic regions called *Cerk* and *Dgke* were associated with a sex-specific acquisition of an anxious phenotype on the elevated plus-maze test. Both regions were named according to the genes that they likely enhance, the ceramide kinase (*Cerk*) and the diacylglycerol kinase (*Dgke*). Both CERK and DGKE enhanced the activity of the Human elongation factor-1 alpha (*EF1*) promoter when cloned into a luciferase reporter construct. Finally, both *Cerk* and *Dgke* were differentially methylated in neurons extracted from micro-dissected ventral dentate gyrus of the brains of mice prenatally exposed to a modified maternal prenatal environment characterized by deficiency in the expression of the serotonin receptor (5-HT_{1a}R). Briefly, that approach involves the transfer of wild-type embryos into the uterus of female mice genetically modified in one of the two functional alleles coding the serotonin receptor 5-HT1AR$^{(+/-)}$. The anxious phenotype affected specifically the male filiation for two generations, and was not genetically acquired since the embryos were those of wild-type mothers transferred to the modified mothers. Similarly, this anxious phenotype was not behaviorally acquired; cross-fostering the pups to wild-types mothers did not erase the anxious phenotype in adulthood [74, 75].

10. PATERNAL STRESS PRIOR TO CONCEPTION MAY EPIGENETICALLY CONTRIBUTE TO INTERGENERATIONAL CHANGES IN BEHAVIOR

In humans and rodents, studies addressing the influence of paternal stress on offspring outcomes *via* epigenetic mechanisms have been very limited as compared to studies of maternal stress. Interestingly, in the fish known as the three-spined stickleback, only fathers take care of the offspring. One study that was performed using that organism showed that paternal care reduced offspring anxiety as well as the expression of Dnmt3a in the whole-brain, responsible for *de novo* DNA methylation [76]. In rats, the impact of paternal stress prior to conception was analyzed on offspring behavior and on the epigenome methylation pattern. Male Long-Evans rats were stressed using an elevated platform during 27 consecutive days before mating and were then compared with controls for behavior of the offspring in the open-field using negative geotaxis tasks and for DNA methylation in the hippocampus and frontal cortex of the brains at P21. Results showed that offspring that were derived from stressed fathers presented delayed task acquisition, reduction of stress reactivity in the open field and reduction of DNA methylation in the frontal cortex among females only, and increased DNA methylation in the hippocampus in both male and female [77]. Importantly, it has to be mentioned that other epigenetic mechanisms may also be involved in the paternal transmission of acquired traits to offspring, such as, for example, microRNAs. In 2014, sperm microRNAs produced by ELS male mice were demonstrated as a putative biological support for a transgenerational transmission of altered behaviors [78]. Using a MSUS mice model, behavioral changes were first observed across generations with reduced avoidance and fear. Additionally, microRNA alterations were observed in male sperm, but also in serum and hippocampus of MSUS derived F1 males. MicroRNA alterations persisted in F2 MSUS mice in serum, hippocampus but no longer in sperm. In contrast, in F3 MSUS animals, microRNA levels returned to control level in all analyzed tissues. The question of whether alteration in sperm-derived microRNAs observed in F1 males can change the behavior of the F2 offspring was tested. In order to answer this question, sperm-derived RNAs from F1 MSUS males were microinjected into wild-type oocytes giving rise to the so-called MSUS-RNAinj condition. That condition was compared to sperm-derived RNAs from F1 control males microinjected into wild-type oocytes, the latter, giving rise to the control-RNAinj condition. Behavioral experiments resulted in the observation that offspring from MSUS-RNAinj condition showed reduced avoidance and fear behaviors as compared with control-RNAinj derived mice. This is consistent with a causal link between altered paternal sperm-derived microRNA or RNA content and altered offspring behaviors.

11. EPIGENETIC STUDIES ON TRANSMISSION OF ACQUIRED TRAITS BY TRAUMATIC HISTORICAL EVENTS

A targeted epigenetic study performed in humans highlighted the transmission of abnormal HPA-axis activity, from mother to offspring, 20 years after initial exposure to a traumatic event. Pregnant mothers that had been exposed to the Tutsi genocide in Rwanda and their offspring were shown to display increased both post-traumatic stress disorder (PTSD) and comorbid depression symptom severity that were associated with increased methylation of the NR3C1 promoter in the blood and decreased cortisol and NR3C1 plasma levels when compared to unexposed mothers and children [79]. Another targeted study involving Holocaust survivors supported intergenerational epigenetic changes in another HPA-axis protein that codes for a gene called *FKBP5* [80]. A genetic polymorphism called rs1360780 located within *FKBP5* gene was shown to confer either GG protective or AG/AA risk alleles for sexual and physical abuses associated PTSD [81]. The polymorphism interacts with epigenetics in the sense that childhood trauma dependent DNA demethylation status impacted differently both alleles. Interestingly, FKBP5 has been reported to interact with NR3C1 as a co-chaperone of the glucocorticoid receptor complex [82].

The "Dutch Hunger Winter" due to the German blockade of food supplies to the Netherlands during World War II, between November 1944 and May 1945, produced a study-cohort of affected pregnant women and their offspring which permitted the study of the effects of prenatal exposure to famine. An untargeted epigenetic analysis was conducted

using whole blood-extracted DNA from exposed individuals that were compared to same-sex siblings born before or after the famine. Across 181 differentially methylated regions which were associated with prenatal exposure to famine, six loci were validated *via* a technically independent method and within independently acquired samples. In two DMRs located in the Carnitine PalmitoylTransferase 1A (*CPT1A*) and in the Insulin Receptor (*INSR*) genes, methylation was shown to induce a decreased expression in Luciferase CpG free plasmids. These two DMRs were significantly associated with birthweight and LDL cholesterol levels [83].

CONCLUSION

New discoveries have emerged recently that elucidate intergenerational transmission of DNA methylation signatures which are associated with ELS. These discoveries have involved both rodent models and untargeted approaches. Further research is needed using animal-models in which there is an attempt to reverse the consequences of ELS *via* an environmentally- and/or pharmacologically based intervention that promotes resilience. Indeed, reversing detrimental ELS consequences would be of great importance for clinical treatment of traumatized human populations. In both rodents and humans, the intergenerational transmission of epi-mutations has been reported, and the mode of transmission seems to be stochastic, meaning that if rules exist, they are unknown. Emerging discoveries of epigenetic regulation of specific neuronal functions, in combination with the current development of functional imaging techniques of dynamic neurotransmission, are beginning to allow for the assessment of specific mechanisms of neural response to ELS. The fundamental question remains with respect to human studies, whether or not the transmission of pathophysiological traits from parents to offspring may be behaviorally acquired after the birth by "education" that is communicated from traumatized parents to children, *versus* biologically transmitted by epi-mutated gametes or, alternatively, by an altered maternal environment during pregnancies. Finally, replication and extension of results that have been obtained in targeted studies performed in rodent and human models *via* new untargeted, genome-wide approaches are strongly needed.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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