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Review

Evidence from clinical and animal model studies of the long-term and transgenerational impact of stress on DNA methylation

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ABSTRACT

While it is well-known that stress during development and adulthood can confer long-term neurobiological and behavioral consequences, investigators have only recently begun to assess underlying epigenetic modifications. In this review, we highlight clinical research and work from animal models that provide evidence of the impact of stressful experiences either during the perinatal period or adulthood on DNA methylation and behavior. Additionally, we explore the more controversial concept of transgenerational inheritance, including that associated with preconception stress experienced by the mother or father. Finally, we discuss challenges associated with the idea of transgenerational epigenetics and for the field of epigenetics in general.

Contents

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1. Introduction

Offspring exist in the context of an ever-changing physical and social environment, and this context plays a huge role in development. Offspring inherit a set of genes from the previous generation, but the operation of this genome is dependent upon environmental context. Stressful events are particularly effective at influencing expressivity of the genome, yielding changes in chemical, anatomical, and neurophysiological properties of the brain that can produce abnormal behavioral trajectories [1–8]. Often, these changes are present long after the initial insult and even in subsequent generations [9]. Therefore, the idea that a stressful environment or experience has the capacity to shape the brain and behavior for the long-haul is now a fundamental principle of neuroscience. More recent research from the field of behavioral epigenetics argues that a mechanism underlying this phenomenon is DNA methylation. This review will describe DNA methylation alterations produced by stress at different developmental time points and functional outcomes and implications of these changes. Further, we discuss transgenerational inheritance of stress-induced DNA methylation alterations, and highlight controversies and challenges for this line of work and the epigenetics field in general.

2. Fundamentals of DNA methylation

The term "epigenetics" was coined by Waddington in the 1940s, referring to the causal interactions between genes and their products which bring a phenotype into being [10]. The term is now traditionally used to describe chemical modifications to chromatin (DNA and its associated histone proteins), and types of epigenetic modifications of this fashion include histone modifications (i.e. acetylation, methylation, and phosphorylation) and DNA methylation and demethylation. In this review, we focus on DNA methylation/demethylation as a mechanism linking stress to behavioral outcomes, including those that span generations.

DNA methylation refers to the addition of methyl groups to cytosines and is catalyzed by a group of enzymes known as DNA methyltransferases (i.e. DNMT1, 3a, and 3b) [11]. Conversely, DNA demethylation refers to the removal of methyl groups and as an active process is thought to occur in one of two ways. The ten-eleven translocation (TET) family of proteins can catalyze oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, leading to a base-excision and replacement with a non-methylated cytosine [12]. Hydroxymethylation has been emerging in the literature as a more stable alteration than previously thought, so it may have more implications for gene activity, neurobiology, and behavior than as a simple intermediate in the demethylation pathway [8,13–15]. Another proposed method of demethylation involves the protein Growth-arrest and DNA damage inducible beta (Gadd45β), which also leads to demethylation via a base-excision repair-like mechanism [16,17].

The addition of methyl groups to CG sites is normally known to repress gene transcription [11]. By directly blocking the binding of transcription factors, mRNA levels decrease. Additionally, methyl groups can bind Methyl-CpG Binding Protein 2 (MeCP2), which recruits histone deacetylases (HDACs) and other co-repressing proteins to repress gene transcription. In a few reported cases, DNA methylation has been associated with active gene transcription (e.g. [18,19]), and instead of always producing basal changes in gene expression, DNA methylation/demethylation are also recognized for their ability to prime transcriptional responses to stimulation [15]. We also point out that studies have found evidence of non-CG methylation in the genome [20,21], which might affect transcription differently than CG methylation [22], and other chemical modifications of cytosines are now known to exist (i.e. 5-formylcytosine and 5-carboxylcytosine), but their functions in regards to behavior are still largely unknown [14]. Finally, while studies have mainly investigated promoter methylation of genes, other work has highlighted the presence of DNA methylation in intragenic regions (i.e. gene bodies) that can likewise alter gene transcription [22].

3. Prenatal stress

3.1. Introduction

Because the brain undergoes tremendous development during the prenatal period, environmental insults can have large-scale and far-reaching effects [23,24]. Though we focus on the effects of psychosocial stress during gestation on offspring DNA methylation, we briefly mention here the effects of two other prenatal stressors with known transgenerational consequences: poor maternal diet and toxin exposure. For example, we have learned of one instance of the connection between fetal experience, long-term negative outcomes, and epigenetics from offspring of parents that experienced the Dutch Famine. These individuals have higher incidences of schizophrenia, depression, and other physical maladies, as well as altered methylation states for a host of genes involved in developmental and metabolic pathways [25,26]. Other empirical reports linking epigenetics to long-term neurobiological and behavioral consequences of poor maternal diet exist [27–31], as is the case for prenatal exposure to a variety of toxins [32–39]. For example, an elegant series of studies summarized in Fig. 1 demonstrate the long-lasting impact of maternal vinclozolin exposure (a fungicide known to disrupt endocrine function) on health and behavior [e.g. 32,33 and many other citations from the Skinner lab].

3.2. DNA methylation in rodents

During gestation (which is 21–23 days in rodents), stressors applied to the mother, such as constant light, wet bedding, noise, frequent cage changes, and restraint, are known to produce long-term changes in her offspring's brain. Early work revealed epigenetic consequences for genes important in stress regulation, including increased methylation of the hippocampal *glucocorticoid receptor* (*GR*) promoter and decreased methylation of the *corticotropin releasing factor* (*CRF*) promoter in the adult amygdala [7]. Both GR and CRF are important in mediating the HPA axis response to stress, and prenatally stressed male offspring also showed maladaptive responses to their own stressful experiences and exhibit depressive-like behaviors [7]. Another mechanism by which prenatal stress may exert negative effects on offspring is through increased methylation of *11β-hydroxysteroid dehydrogenase type 2* (*HSD11B2*), which normally protects the fetus from maternal glucocorticoids by converting them to inactive metabolites [40].

Work with a prenatal restraint stress model known to produce neural and behavioral characteristics reminiscent of schizophrenia, in which pregnant dams are placed in a plastic tube twice a day for gestational days 7–21, has shown increased cortical levels of DNMTs, methylation, and hydroxymethylation of *brain derived neurotrophic factor (Bdnf) IV*, *reelin*, and *GAD67* genes in offspring, all of which parallel decreases in mRNA [8,41]. Schizophrenia symptomatology in this model is reversible with an HDAC inhibitor or clozapine, both of which have demethylating effects [42]. Stress-induced *Bdnf IV* methylation has also been observed in another model that limited prenatal stress (variable stress paradigm, including restraint and swim stress) to the third week of gestation, whereby offspring had increased *Bdnf IV* methylation in the amygdala at postnatal day (PN) 21 and 80

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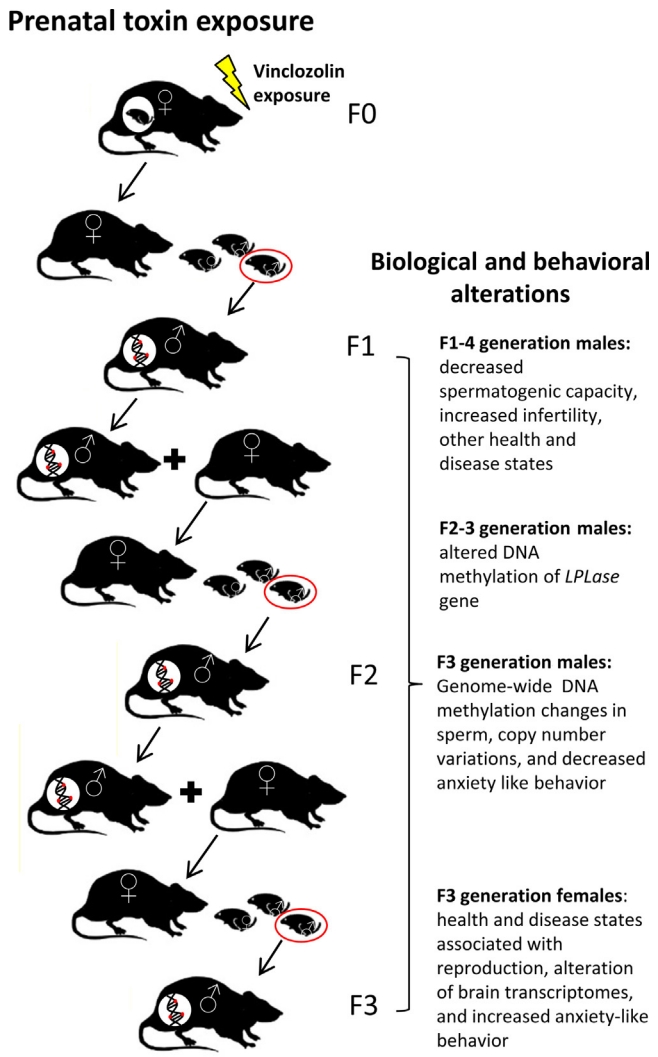

Fig. 1. Maternal exposure to the fungicide vincozolin during pregnancy has transgenerational effects on offspring health and behavior. When males are exposed to vinclozolin in utero, they exhibit deficits in reproductive function and altered disease states, effects that are present in many generations post-exposure and passed through the germline (sperm DNA).

and increased hippocampal methylation at PN80 [43]. Together, these studies demonstrate the long-term effects of fetal stress on adult methylation patterns, with gene targets including those involved in development, stress responsivity, and many psychiatric disorders.

3.3. DNA methylation in humans

Maternal depression is recognized for its lasting effects on offspring health, and epigenetic consequences perhaps responsible include increased methylation of the *GR* gene *(Nr3c1)* [44,45]. Suggesting functional relevance, such methylation changes have been shown to correspond to an increase in salivary cortisol provoked by a stress challenge when infants are 3 months of age [44]. While maternal emotional state seems to be a predictor of infant *GR* methylation levels and stress response, other stressors during pregnancy can serve as a catalyst for long-term changes in offspring methylation. For example, adolescents of mothers who experience domestic violence during pregnancy have increased methylation of the *GR* promoter [46]. Further, genome-wide changes in methylation, mainly of genes associated with immune function, were found in thirteen year old children of mothers who were pregnant during the 1998 Quebec ice storm [47]. Taken together, these studies demonstrate the strong effects of both maternal emotional state and adversity experienced during gestation on offspring methylation.

4. Early-life postnatal stress

4.1. DNA methylation in rodents

It was originally thought that DNA methylation only took place during cell development and differentiation (i.e. before birth), but work continues to defy this concept and demonstrates epigenetic consequences of stress outside of embryonic development. Capitalizing on natural variations in maternal care, investigators have shown maternally-driven *GR* methylation patterns in offspring. Specifically, rats that experience low levels of maternal care (low licking/grooming (LG)) during the first week of life show increased methylation of *GR* in the hippocampus that is present during infancy and persists through adulthood, decreased *GR* mRNA/protein, and increased corticosterone levels in response to a stressor [48]. Behavioral and epigenetic effects of low LG are reversible by cross-fostering to high LG dams or by treatment with an HDAC inhibitor [48]. Further, high vs. low levels of maternal care can be passed on to offspring such that high LG offspring became high LG dams themselves, which involves epigenetic programming of the estrogen receptor in females [49]. Maternal care-induced changes in methylation also extend to other transcriptional and intragenic sequences that alter gene activity [22].

Another model of early-life stress involves the absence of maternal care altogether for discrete periods of time. Offspring which experience maternal deprivation (3 h/day for the first 10 postnatal days) show a long-lasting decrease in methylation of hypothalamic *arginine vasopressin* (*AVP*) and pituitary *propiomelanocortin* (*Pomc*), two gene loci also important in the HPA axis response to stress [50,51]. Further, these offspring show increased *AVP* and *Pomc* gene expression and hypersecretion of corticosterone, which parallel behavioral deficits in stress coping and memory [50,51]. Another gene involved in the stress response, *CRF*, is also sensitive to maternal separation. Specifically, there is converging evidence that maternal separation elicits hypomethylation of hypothalamic (paraventricular nucleus, PVN) [52] and hippocampal [53] *CRF* as well as cortical and sperm *CRF receptor 2* (*CRFR2*) methylation in animals [54]. Such methylation changes parallel both increases in gene expression and behavioral deficits, including memory deficits [53], synaptic dysfunction [53], depressive-like behaviors [54], and HPA-axis hypersensitivity [52]. These methylation and behavioral abnormalities have been found to even extend into the next generation [54]. Finally, the far-reaching effects of maternal separation can also involve methylation of the hippocampal *retinoic acid receptor* (*RAR*) promoter, a gene important in differentiation of neural progenitor cells [55], suggesting a role of early-life stress-induced methylation in neurogenesis.

Our lab utilizes another model of early-life stress in which rat pups experience an abusive and neglectful caregiver for 30 min a day for the first week of life [56–59]. Characteristic maternal behaviors experienced by pups in our maltreatment condition include stepping on, actively avoiding, and roughly handling pups. Using this model, we have characterized multiple long-term epigenetic effects of early-life stress on *Bdnf* DNA. Foundational studies demonstrated that rats that experience maltreatment have increased methylation in the whole PFC [59]. Alterations in *Bdnf* methylation were subsequently detected in the medial prefrontal cortex (mPFC), amygdala, and hippocampus with increases or decreases varying by region, demonstrating the brain

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region-specific nature of stress-induced changes in *Bdnf* methylation [56–58]. Notably, in the whole PFC, the increase in methylation for one region of the *Bdnf* gene (exon IX) was present in infancy (24 h after the last caregiver manipulation) and persisted through adolescence and into adulthood (PN90) [59]. Further, not only were PFC methylation changes long-lasting in the generation that experienced the mistreatment, but the next generation showed similar *Bdnf* methylation patterns as well [59]. Due to the importance of *Bdnf* in development and synaptic plasticity and its implication in multiple psychiatric disorders, long-lasting and transgenerational effects of early-life stress on methylation of this gene provides a possible mechanism for psychopathological outcomes associated with maltreatment.

4.2. DNA methylation in humans

While post-mortem studies of brain tissue are rare, the few studies that exist are extremely informative. There is higher *NR3C1* (*GR*) promoter methylation in post-mortem hippocampal tissue from adult suicide victims who had experienced childhood abuse, a finding that parallels rodent work [60,61]. Genome-wide methylation changes have also been found in hippocampal neurons of this cohort [62,63].

Because port-mortem samples are difficult to acquire, a large body of work has focused on peripheral measures of DNA methylation. Blood samples from children that experienced institutionalization during infancy show genome-wide methylation, mainly in genes related to immune and cellular signaling pathways [64]. Genome-wide changes in methylation have also been found in buccal cells of adolescents that were subjected to high levels of maternal or paternal stress in infancy and early childhood [65]. Other studies using peripheral measures in humans have emphasized the long-term effects of early-life stress in specific genes important for stress responsivity and implicated in psychiatric disorders. For example, increased *NR3C1* promoter methylation was found in leukocytes of adults that reported experiencing stress or neglect early in life, which corresponded to an attenuated cortisol response during a stress challenge [66]. Severity of childhood sexual abuse has been shown to positively correlate with *NR3C1* methylation in adults with Borderline Personality Disorder [67], and more recent work with this cohort has revealed genome-wide methylation alterations [68].

Another gene involved in long-term outcomes in stress reactivity is *FK506 binding protein 5* (*FKBP5*). Measures in peripheral blood of subjects diagnosed with PTSD have elucidated a link between genotype, early-life stress, and susceptibility for PTSD following later trauma. Specifically, a functional polymorphism in the *FKBP5* gene, in conjunction with childhood trauma and demethylation, increase susceptibility for PTSD [69]. The *serotonin transporter gene* (*5HTT*), which plays a crucial role in depression, also exhibits differential methylation after early-life stress. In women, methylation at this gene locus was associated with self-reported sexual abuse during childhood and was also associated with symptoms of antisocial personality disorder during adulthood [70,71]. Genome-wide hypermethylation has been observed in males with depression who experienced separation from parents in early-life, especially for genes involved in brain development [72]. Finally, work has shown substantial differences in genome-wide methylation and gene expression patterns in individuals exposed to maltreatment in childhood verses those not exposed [73]. In conjunction with the animal research described earlier, these studies highlight the strong susceptibility of the epigenome (particularly of genes involved in development, plasticity, stress responsivity, and immune pathways) to methylation alterations following early-life trauma, which in turn may produce vulnerability to psychopathology.

5. Adult stress

5.1. DNA methylation in rodents

An even more recent advancement in the field of epigenetics is the presence of behaviorally- and stress-mediated DNA methylation or demethylation in adult animals (those that have reached sexual maturation). Whether such epigenetic changes are as long-standing (months to years) as those observed with developmental stress has not been empirically addressed, but as will be discussed in the next section, the transgenerational nature of preconception stress underscores the likelihood of such long-term changes.

In rodents, stress during adulthood is known to influence methylation of various genes in the brain. Exposure to chronic and unpredictable stressors, including cage tilt, confinement, and reversed light cycles, produces hypermethylation of the *glial cell-derived neurotrophic factor* (*Gdnf*) gene in the nucleus accumbens, which is accompanied by decreased mRNA [74]. Multiple groups have used a social defeat paradigm to model chronic social stress, in which one mouse is "bullied" by another for multiple consecutive days during adulthood. One group found that chronic social defeat stress causes demethylation of the *CRF* gene in the PVN, an effect present for at least two weeks and corresponds with the development of social avoidance behavior [75]. Other groups have found alterations in levels of *DNMT3a* (nucleus accumbens) following social defeat stress, which are reversible by a DNMT inhibitor [76]. Moreover, mice that are resilient to social defeat stress (show less social avoidance following stress) have been found to have a higher density of DNMT3a type II cells in the hippocampus which reflect newly differentiated neurons, implicating methylation and neurogenesis in stress-buffering effects [77].

Rodent models of PTSD likewise illustrate the ability of stressful events to affect DNA methylation in the brain. In one model, rats were placed on well-soiled cat litter for 10 min to simulate a traumatic experience [78]. After 7 days, stressed rats had decreased methylation of the hippocampal *Disks-Large Associated Protein* (*DIgap2*) gene (which codes for a post-synaptic density protein) and increased mRNA expression, an effect that was related to the strength of startle response exhibited during behavioral testing [78]. In another model, restrained rats were exposed to the presence of a cat in conjunction with social instability (i.e. different cagemates each day). Rats that experience this trauma show cognitive and emotional deficits relevant to those found in human subjects with PTSD, which could possibly be mediated by long-term epigenetic changes [79]. Several weeks after psychosocial stress, rats showed altered methylation patterns of *Bdnf* in different subregions of the hippocampus, which correlated with altered *Bdnf* mRNA expression [80]. Changes to gene methylation have also been elicited by fear conditioning and extinction paradigms [81–83] (see Blaze and Roth, 2011 for a comprehensive review of epigenetic mechanisms in learning and memory).

5.2. DNA methylation in humans

Many clinical studies have used combat veterans to link epigenetics and PTSD. Genes involved in immune function in particular have been implicated in PTSD, and multiple groups have found changes in DNA methylation of these genes resulting from traumatic experiences [84–86]. The repetitive genomic elements *line-1* and *Alu* [87] exhibit changes in methylation following trauma exposure, and work has also highlighted the link between traumatic events and *SLC6A4* [88] and *GR* methylation[89]. Further, some studies suggest that methylation changes actually play a role in resiliency to PTSD [87,88], an intriguing concept that warrants further research.

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The Trier Social Stress Test (TSST) is a commonly used procedure to induce psychosocial stress in human subjects within a laboratory setting. In this test, subjects must perform mathematical calculations and give an oral presentation in front of a panel of "experts" [90]. A handful of studies have reported DNA methylation changes resulting from the TSST. For example, the *oxytocin receptor* gene (*OXTR*), a gene highly involved in social behavior and stress, was hypermethylated in one group of individuals following the TSST [91]. Changes in *OXTR* methylation following the TSST was likewise reported in patients with social anxiety [92], and another group found that *5HTT* methylation moderates the association between a polymorphism at this gene locus and cortisol stress reactivity after the TSST [93]. Collectively, these studies indicate that stress during adulthood has epigenetic consequences, with relevance for stress-related disorders including PTSD.

6. Transgenerational inheritance of epigenetic marks

6.1. Introduction

We have discussed the effects of early-life and adult stressors on DNA methylation and behavior. We have briefly mentioned transgenerational effects of DNA methylation following developmental stress when appropriate, but here we delve further into this phenomenon. Information on transgenerational inheritance in terms of epigenetics has been more thoroughly investigated in plants and invertebrates [94]. In plants, epigenetic silencing via DNA methylation mainly occurs in repetitive DNA, transposons, and transgenes, and does not preferentially occur at CG dinucleotides [95,96]. Additionally, unlike mammals, the majority of methylation takes place within the gene body, with minimal methylation at the promoter region [95,96] The plant literature has numerous examples of transgenerational inheritance of phenotype after stress (see Hauser et al., 2011 for a comprehensive review). This literature also suggests that a mechanism driving transgenerational epigenetic changes could be the lack of erasure of some epigenetic marks from the genome during reproduction [95]. Though it is well-established for the mammalian genome that there are sequential and large-scale epigenetic changes, including genome-wide DNA demethylation and imprint erasure, which help reset the epigenome between each generation and restore totipotency [97–99], there is evidence however that some methylation escapes the reprogramming process [98,99].

An emerging concept in the behavioral epigenetics field is that preconception stress, or experiences that a parent (F0 generation) had earlier in life, produce alterations in epigenetic and gene expression states and behavior in offspring and grand-offspring (F1–F2 generations) (Fig. 2). The idea of transmission of phenotype across generations is not novel, but the idea that epigenetics may mediate this transmission and the extent to which this occurs in humans is debated [100–104].

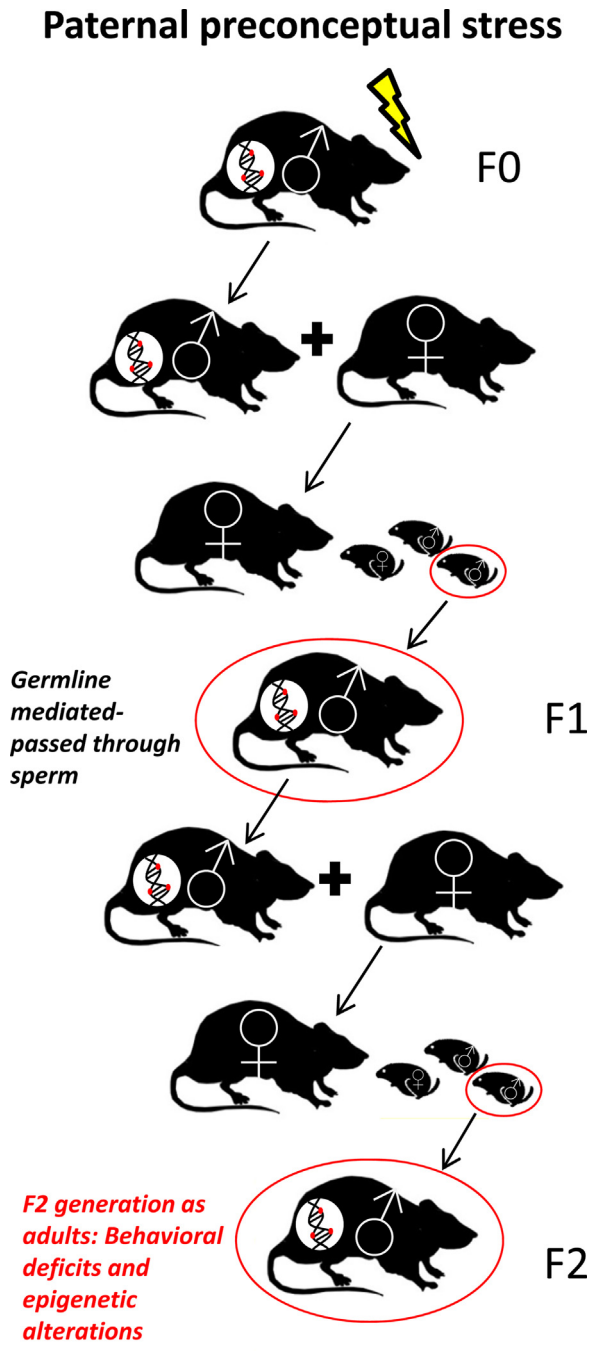

Fig. 2. Examples of germline-mediated transgenerational inheritance. Paternal stress experienced before conception can alter DNA methylation and phenotype for several generations, an effect that is passed through the male germline.

6.2. Preconception stress in rodents

Studies have shown behavioral and neurobiological abnormalities in offspring following preconception stress of the mother. For example, mild preconception stress of females (foot shock, swim stress, and elevation on a plexiglass platform) 2 weeks prior to conception produces behavioral alterations in offspring that last well into adulthood. Possible neurobiological correlates of these behavioral deficits include changes in neuronal morphology in the mPFC of offspring [105], but whether these transgenerational outcomes are conferred by stress-induced changes in maternal behavior or germ-line epigenetics (see *Modes of transmission* below [106]) is not known. One recent study has provided empirical support that preconception stress can epigenetically modify the germ-line (and brain) of stressed female rats. Specifically, in both mature oocytes and frontal cortices of females that underwent chronic unpredictable there was increased *CRF1* mRNA [107]. Further, their offspring also had increased *CRF1* mRNA in brain tissue [107].

Perhaps more surprising is the observation that paternal preconception stress can influence offspring development, especially considering the fact that rodent fathers are typically removed from the cage after conception (since they are not necessary for care of the offspring). Males that experienced maternal separation during infancy have been shown to pass on their depressive-like phenotype to offspring and grandoffspring [54]. These males have altered methylation of *MeCP2*, *cannabinoid receptor 1* (*CB1*), and

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corticotropin releasing factor receptor 2 (*CRFR2*) in their sperm, and their offspring have similar methylation patterns in brain tissue [54]. Offspring born to male mice who were exposed to social defeat stress show increased anxiety- and depressive-like behaviors [108]. Notably, some transgenerational effects can be produced through IVF (with sperm from the stressed male), while others are abolished.

Other groups have likewise found epigenetic changes associated with paternal stress prior to conception. In one study, males were stressed for 27 consecutive days by being placed on an elevated Plexiglas platform, and subsequently their 21-day old offspring were found to have behavioral deficits and altered global methylation in the frontal cortex and hippocampus [109]. Another group administered synthetic glucocorticoids to males 60 days prior to conception, and found global increases in non-CG methylation in their sperm [110]. In the F1 generation, adult males had significant differences in mRNA expression levels of various stress-related genes in the kidney and hippocampus, with demethylation of these genes in the kidney [110]. These results suggest that glucocorticoids produced by environmental stress before conception play a role in the transgenerational epigenetic effects. In one final study we highlight here, investigators conditioned mice to fear an odor (acetophenone) that activates a known olfactory receptor (*Olfr151*), and found that F1 and F2 generation offspring exhibited enhanced sensitivity to that odor when tested [111]. Additionally, offspring showed an enhanced neuroanatomical representation of the *Olfr151* pathway and hypomethylation of the *Olfr151* gene, and neither IVF or cross-fostering could erase the epigenetic effects of exposure to the odor [111].

6.3. Preconception stress in humans

Though far less empirically studied in humans, there is some evidence of the epigenetic transmission of the Holocaust trauma on survivors' children. Some earlier work had found no link between parents' experience in the Holocaust and offspring behavioral outcomes [112,113], but a more recent study has found far-reaching epigenetic outcomes in Holocaust survivor offspring [114]. Specifically, offspring who had a father with PTSD following the Holocaust had increased methylation of the *GR* promoter. Offspring whose parents both had PTSD had lower levels of *GR* methylation, and their decreased *GR* methylation was also associated with suppression of cortisol after a stress challenge. Together, these data demonstrate stress-related epigenetic and behavioral effects of preconception stress in humans, and that there may be different mechanisms for the intergenerational transmission of Holocaust trauma.

7. Challenges in epigenetics and exciting opportunities for future research

7.1. Modes of intergenerational transmission

One question that arises from review of these studies is whether transmission takes place due to behavioral mechanisms (i.e. driven by maternal care or other social cues) or epigenetic alterations that are being transferred through the germline. For example, the transgenerational impact of low levels of maternal care on offspring phenotype and *GR* methylation are reversible by cross-fostering, indicating these transgenerational effects are behaviorally-mediated through maternal care [48]. Our lab has shown that *Bdnf* methylation induced by maternal maltreatment can be passed across generations, but cross-fostering could not completely erase these epigenetic marks, suggesting germ-line inheritance. Our observations however of anxiety-like behavior in these females prior to parturition cloud this issue, as maternal state during gestation could have also influenced methylation patterns [59].

For germ-line inheritance, the epigenetic marks must be present in the germline and not cleared between generations. Some studies provide empirical support that this can occur [54,107,111], but many studies have not identified the mode of transmission. It stands to reason that future studies that incorporate methodology (such as IVF and cross-fostering) to tease out behaviorally-mediated vs. germ-line-mediated inheritance, will reveal substantial information regarding *how* paternal traumatic experiences affect offspring and grand-offspring.

7.2. Cell-type and brain-region specificity

Another challenge for the field, and one that we have not touched upon until here, is cell-type specificity, both between the CNS and periphery and that within the brain. Epigenetic modifications present in the periphery often differ from those in the brain due to differences in cell type (blood or saliva vs. neurons and glia) [115]. Recent work has highlighted saliva as a slightly more suitable match to brain samples (instead of blood), perhaps due to increased epithelial cells present in saliva [115]. Furthermore, within the brain, neurons and glia (astrocytes, oligodendrocytes, microglia) can exhibit distinct methylation patterns, and there is evidence for region-specificity of stress-induced alterations. The differential effect of cell composition on DNA methylation suggests the benefit of cell-sorting in future epigenetic studies [116,117], and the issue of brain region-specificity is an important point to consider in the context of comparing epigenetic alterations in the periphery with epigenetic alterations in the brain.

7.3. The new cytosine variants

The final challenge we highlight is the need to distinguish 5-methylcytosine from other cytosine variants (i.e. 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine) that have come to light. While hydroxymethylation is suggested to play a role in DNA demethylation, functions of this molecular mark and the other "flavors" of DNA methylation are still largely unknown. The conventional methods used for mapping 5-mC (i.e. bisulfite treatment and sequencing) do not differentiate it from 5-hmC. Standard bisulfite treatments convert unmethylated cytosines to uracil for downstream methylation analyses, but this method does not distinguish between methylated and hydroxymethylated cytosines. Recent protocols have been generated to address this (e.g. [118]), and such approaches can help elucidate the role of these multiple cytosine variants in gene expression, brain function, and behavior.

8. Conclusions

Overall, we have highlighted several lines of evidence for epigenetic modification of genes relating to stress and psychopathology linking behavioral outcomes to developmental and adult stress. It is clear that stress during the lifetime can leave its mark through epigenetic modifications, and can do so not just in the generation directly experiencing the stress, but in subsequent generations as well. There is still much to be discovered in the field of epigenetics in relation to behavior, psychiatric disorders, and transgenerational epigenetic inheritance, rendering many opportunities for fascinating new research.

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