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Review

The effects of early life stress on the epigenome: From the womb to adulthood and even before

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ABSTRACT

Exposure to early life stress (ELS), such as childhood abuse and neglect is a well established major risk factor for developing psychiatric and behavioral disorders later in life. Both prenatal and postnatal stressors have been shown to have a long-lasting impact on adult pathological states where the type and timing of the stressor are important factors to consider. There is a growing body of evidence suggesting that epigenetic mechanisms play a major role in the biological embedding of ELS. A number of studies now indicate that the epigenome is responsive to external environmental exposures, including the social environment, both during intra-uterine development and after birth. In this review, we summarize the evidence of long-lasting effects of ELS on mental health and behavior and highlight common and distinct epigenetic effects of stress exposure at different stages during development. These stages include postnatal stress, prenatal stress, i.e. in utero and stress occurring pre-conception, i.e. effects of stress exposure transmitted to the next generation. We also delineate the evidence for the possible molecular mechanisms involved in epigenetic programming by ELS and how these maybe distinct, according to the timing of the stress exposure.

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Introduction

The early life is one of the most important and sensitive periods during the development of an individual (Lupien et al., 2009). At this stage, the body and especially the brain are known to be greatly responsive to environmental cues since they undergo dynamic changes (Bock et al., 2014). Early life stress (ELS) has been associated with a wide range of health problems later in life such as increase reactivity to stress, cognitive deficits, psychiatric and behavioral disorders (Heim and Binder, 2012; Loman et al., 2010; O'Connor et al., 2005). Both prenatal and postnatal stressors have been shown to have a long-lasting impact on adult psychopathology where the type and timing of the stressor as well as gender, are important moderating factors (Heim and Binder, 2012).

ELS includes various types of stressor that can occur as early as the prenatal period and up to adolescence. In humans, ELS during pregnancy

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include stressors such as exposure to malnutrition, exogenous glucocorticoids and maternal depression or anxiety whereas postnatal stressors include exposure to maternal postpartum depression or anxiety, child abuse and or neglect, poverty, loss of a parent and exposure to family conflict and violence, all of which have been shown to lead to increased risk for psychiatric disorders in adulthood. In the last decade, there is increasing evidence that epigenetic marks are likely to play a major role in the molecular mechanisms underlying the long-lasting effect of ELS on adult health. Indeed, there is a growing body of evidence suggesting that in addition to its role in cellular programming, the epigenome is also responsive to external environmental exposures including the social environment both during intra-uterine development and after birth in animals (Darnaudery and Maccari, 2008; Gudsnuk and Champagne, 2012) and in humans (Klengel et al., 2014; Mill et al., 2008; Sasaki et al., 2013; Szyf, 2012). This would allow to prime future responses of an organism, depending on its early environment.

In this review, we will first describe some of the evidence of the long-lasting effect of ELS on mental health in humans and behavior in animal models. We will illustrate examples where long-lasting epigenetic alterations have been shown to associate with ELS at different stages during development starting with postnatal stress followed by prenatal stress, i.e. in utero and pre-conception stress defined as stress occurring in the previous generation and transmitted to the next generation. Moreover, we will attempt to delineate the possible mechanisms involved in epigenetic programming of ELS and how these may be distinct, depending on the timing of ELS.

Overview of the epigenome

The main function of the epigenome is to regulate gene transcription and compaction of the DNA into the cell nucleus. Several distinct epigenetic marks come together to achieve this, including DNA methylation and hydroxymethylation, histone modifications, ATP-dependent chromatin remodeling and non-coding RNAs. Briefly, post-translational modifications of histone tails, such as acetylation, methylation and phosphorylation can be classified as either transcriptionally activating or repressing marks depending on the resulting effect on the compaction of DNA and the recruitment of other protein complexes such as transcription factors and repressors. Alterations in histone acetylation and methylation status have been shown to be induced by adverse environments in animals (Tsankova et al., 2006; Weaver et al., 2004a, 2004b). However, it is believed that since these marks are more transient than other epigenetic modifications, they cannot fully explain the long-term and transgenerational effects of the environmental programming observed following ELS. Interestingly, this hypothesis has been recently challenged by a study by Brunner et al. revealing histone modifications in the sperm that can be stabilized in protamine marks and transmitted to the next generation through the germ line (Brunner et al., 2014). Nonetheless, most studies on the epigenetics of early life stressors in the past have focused on DNA methylation and microRNAs.

DNA methylation is a covalent modification of the cytosine residues that are located primarily but not exclusively at CpG dinucleotide sequences in mammals (Lister et al., 2009; Xie et al., 2012). Increased DNA methylation in the promoter region or in the first exon is usually associated with repressed gene expression (Bird, 1986) whereas DNA methylation located within the gene body, enhancer and intergenic regions (Ball et al., 2009; Lister et al., 2009; Maunakea et al., 2010; Ogoshi et al., 2011) is found to correlate both negatively and positively with gene expression (Jiang et al., 2013; Jjingo et al., 2012; Mehta et al., 2013). In addition to its role in gene expression, genomic DNA methylation also plays an important role in the maintenance of genome integrity and heterochromatin formation (Bestor, 1998; Hedges and Deininger, 2007; Miniou et al., 1994).

The main purpose of DNA methylation and other epigenetic marks is to confer cell-specific gene expression identity that is formed during embryonic development (Lister et al., 2009; Razin and Szyf, 1984; Vire et al., 2006). To accurately maintain the DNA methylation profiles and prevent a drift in the DNA methylation pattern during the life course, several biochemical elements come into play. DNA methyltransferase 1 (DNMT1) maintains the methylation pattern during cell division by copying the parent strand (Bestor, 1998) and DNMT3a and 3b are responsible for active DNA methylation by de novo methylation (Okano et al., 1998). In addition to passive demethylation with cell division, several processes have now been proposed to be responsible for active DNA demethylation, mostly in response to environmental triggers. These include direct action of a demethylase that removes the methyl group from the DNA (Ramchandani et al., 1999) as well as more complex DNA repair-based mechanisms (Barreto et al., 2007; Ma et al., 2009; Morgan et al., 2004; Rai et al., 2008; Schmitz et al., 2009). The more recently discovered 5-hydroxymethylcytosine in stem cells and neurons is proposed to serve as an intermediate modification of 5-methylcytosine, which is catalyzed by ten-eleven translocation (TET) proteins (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009), and leads to replacement by an unmethylated cytosine through nucleotide and/or base excision repair factors (Guo et al., 2011a, 2011b). Hydroxymethylation may not only serve as an intermediate step in active demethylation, but was also proposed to alter gene expression on its own by recruiting transcription regulators (Ficz et al., 2011; Jin et al., 2010; Mellen et al., 2012; Spruijt et al., 2013) or through binding of TET1 (Zhang et al., 2010a, 2010b, 2010c) and/or TET3. Hydroxymethylation was also shown to dynamically mediate behavioral adaptations (Li et al., 2014).

Another component of the epigenome is the non-coding RNA (ncRNA). ncRNAs were shown to regulate genes translation and transcription as well as chromatin stability (Zhou et al., 2010). Such ncRNAs include short microRNAs, PIWI-interacting RNAs and long non-coding RNAs. ncRNAs can regulate gene expression through post-transcriptional binding to the 3′UTR of mRNA (Filipowicz et al., 2008), by directly binding to promoters and interfering with polymerases (Wassarman and Storz, 2000), or by localizing transcriptionally repressive complexes onto the heterochromatin (Zaratiegui et al., 2007).

It is important to keep in mind that even if these epigenetic components are often studied separately, they interact to exert a combined modulation of gene transcription/translation. These marks can prime not only current but also future gene expression and translation modifications. The true nature of such relationships can only be revealed if longitudinal as well as intergenerational epigenetic studies are conducted in parallel.

Postnatal stress

The importance of postnatal stressful environments on child development is well illustrated in a study performed by Kolominsky et al. who investigated infants whose mothers were exposed to radiation during pregnancy in the atomic accident of Chernobyl in 1985. Surprisingly, researchers found that not the level of exposure to radiation, as anticipated, but rather the mothers' and fathers' level of stress due to evacuation and relocation best explained the infants' psychological symptoms (Kolominsky et al., 1999). While childhood adverse life events include a number of factors ranging from early parental loss to low socioeconomic status, the most profound long-term impact is seen in individuals exposed to childhood abuse and/or neglect. In fact, reports from the World Health Organization (2002) indicate that about 20% of women and 5–10% of men are exposed to sexual and or physical child abuse and that an approximately 20% of children are neglected worldwide. Child abuse and neglect have been associated with increase risk for developing a range of psychiatric disorders later in life such as mood and anxiety disorders (Heim and Nemeroff, 2001), PTSD (Bremner et al., 1993; Widom, 1999) and antisocial behavior (Rutter, 1998; Widom, 1989). The early programming of systems involved in emotion and stress regulations seem to mediate this increase risk later in life.

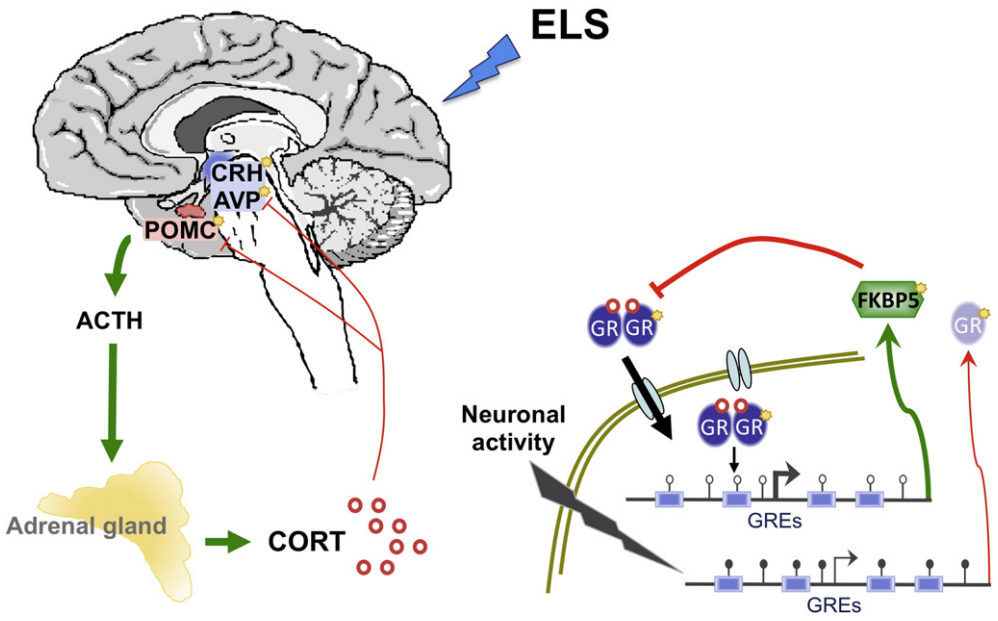

Fig. 1. Epigenetic alterations induced by early life stress (ELS) in genes regulating the hypothalamic–pituitary–adrenal (HPA) axis increase the stress response. This figure briefly summarizes the activation of the HPA axis by stress. The neuropeptides corticotropin releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus and activate the transcription of pro-opiomelanocortin (POMC) gene, then cleaved into adrenocorticotropic hormone (ACTH) which is released from the pituitary and activates the secretion of cortisol (CORT) from the adrenal gland. Cortisol binds to the mineralocorticoid receptor (MR) as well as the glucocorticoid receptor (GR). Upon hormone binding, the receptor complexes translocate into the nucleus to regulate the transcription of various genes involved in the stress response. This transcriptional activation may also trigger DNA demethylation within and around glucocorticoids response elements (GREs) (Thomassin et al., 2001). The activation of the GR is critical in initiating a negative feedback loop responsible for terminating the stress response and therefore the secretion of cortisol. A number of studies have shown that ELS can lead to epigenetic changes in key regulators of the stress-response, thereby altering the set-point of the HPA-axis. For example, ELS-induced DNA demethylation of the *FKBP5* gene increases its activation by GR activation and by reducing GR sensitivity impairs the negative feedback regulation and prolongs the cortisol response (Klengel et al., 2013). ELS can also trigger DNA methylation of the *NR3C1* gene through neuronal activation also leading to an impaired negative feedback by reducing GR level (Weaver et al., 2004a, 2004b; Weaver et al., 2007). In addition, ELS-induced alterations in DNA methylation of the *CRH* (Elliott et al., 2010) and *AVP* (Murgatroyd et al., 2009) genes can increase/prolong the stress response by altering the secretion of ACTH, but also by changing the transmission of these neuropeptide in limbic brain regions. Genes shown to be epigenetically altered or associated with ELS are marked with a yellow star. On the right is a schema of a cell nucleus depicting GR regulated genes with their regulatory region and/or GREs as being either methylated (bottom) or unmethylated (top). This can be induced by either GR binding or neuronal activation after exposure to ELS and leads to changes in gene transcription.

Therefore, the most investigated hypothesis on how ELS can alter the child's development in utero and postnatally is that this is mediated by long-term effects of on the function an activity of genes involved in the stress hormone or hypothalamic–pituitary–adrenal (HPA) axis (see Fig. 1). Upon activation of the HPA axis, corticotropin releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus and stimulate adrenocorticotropic hormone (ACTH) release from the pituitary into the blood. This results in glucocorticoid (cortisol in humans and corticosterone in rodents) secretion from the adrenal cortex. The main features of the HPA axis are a basal circadian activity rhythm, a negative feedback mechanism moderated by corticosteroids that limit the response of the axis after its activation, and molecular and cellular differences in the response to acute vs. chronic stress. These actions are mediated through binding to the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) that act as transcription factors and are expressed in most tissues (Larsson et al., 2012; Sanchez et al., 1993). Binding of cortisol to the GR and MR induces their translocation into the nucleus where they can exert their function as transcription factors regulating adaptive responses to stress, including metabolism, immune activation and cell proliferation and differentiation. At multiple levels of the HPA axis, the activation of the GR will initiate a negative feedback loop that is responsible for terminating the stress response and therefore the secretion of cortisol. A decrease in GR expression/activation is generally associated with an increase in the response to stress due to an impaired negative feedback.

Dysregulation of the HPA axis is one of the most robust findings in biological psychiatry. Since the HPA axis is one of the main systems activated after exposure to a stressor, genes regulating this system are prime candidates for epigenetic research on the biological embedding of ELS. The following section will discuss evidence for associations of epigenetic alterations with long-lasting effects of postnatal stress with a focus on key regulator genes of the HPA axis.

Evidences for epigenetic reprogramming following postnatal stress

In animal models, various types of ELS such as maternal separation and maternal stress have been studied to investigate mechanisms of action of long-lasting effect of stress on adult phenotype and their mediation by epigenetic alterations (see (Darnaudery and Maccari, 2008; Gudsnuk and Champagne, 2012) for review). Similar to humans, mice subjected to early postnatal stress display a range of behavioral alterations depending on the type of stressors and their length. Such phenotypic alterations included higher stress reactivity, higher anxiety- and depressive-like phenotype and addiction (see (Cheng et al., 2013) for review). In brain tissue, DNA methylation was found to be altered following postnatal stress in genes regulating the HPA axis including the gene encoding the GR (*Nr3c1*) (Weaver et al., 2004a, 2004b), the hypothalamic peptides *Avp* (Murgatroyd et al., 2009) and *Crh* (Elliott et al., 2010), and their receptors, *Crhr2* (Franklin et al., 2010). Some of these changes were even maintained into adulthood. These epigenetic alterations are thought to mediate the altered response to stress seen in these animals via persistent changes in gene expression. Postnatal stress was also shown to affect not only the stress response but also neuronal development in ELS exposed animal models. This may be mediated by altered gene expression profile of neurotrophic genes such as *Bdnf* (Roth et al., 2009) and *Gad1* (Zhang et al., 2010a, 2010b, 2010c) that were accompanied by DNA methylation changes.

Parts of these results have also been translated to humans where brain studies could be performed on post-mortem samples. The state of methylation and expression of the *NR3C1* exon 1_F alternate transcript was examined in the hippocampus of suicide victims (McGowan et al.,

2009). Site-specific differences in DNA methylation in the *NR3C1* promoter and its expression were detected between suicide completers who had reported child abuse versus those who had not. These changes were not only restricted to the *NR3C1* gene. Analysis of the DNA methylation status in a large genomic locus containing the *NR3C1* gene identified numerous regions such as the *protocadherin* (*PCDH*) gene cluster, that displayed differences in DNA methylation associated with both maternal care in rats and child abuse in humans (Suderman et al., 2012). Moreover, genome-wide studies identified differential DNA methylation in many gene promoters in the hippocampus involved in cellular/neuronal plasticity associated with severe childhood abuse (Labonte et al., 2012).

In contrast to animal studies and a very limited number of human studies, most of the clinical and epidemiological work currently ongoing does not include brain samples. Thus, epigenetic studies in humans have used peripheral tissues such as blood and buccal cells and identified numerous epigenetic associations with early life stress and adversity. These associations can be seen as simple biomarkers but might also be causally involved in the disease state through their action on the HPA axis and immune system for example (Miller et al., 2005).

In a recent study, we analyzed gene expression and DNA methylation profiles in peripheral blood cells among individuals with PTSD, with or without prior exposure to severe child abuse. We identified distinct patterns of gene expression and a higher contribution of DNA methylation underlying gene expression differences among patients with a history of childhood abuse (Mehta et al., 2013). This suggests differential biological mechanisms associated with PTSD-dependent gene expression differences according to the time and severity of the environmental exposure. A study by Suderman et al. also identified a specific DNA methylation signature in peripheral blood associated with childhood abuse, where differential methylation was also observed in promoter regions of loci encoding microRNAs. This may suggest that additional epigenetic mechanisms could be involved in the biological embedding of postnatal stress (Suderman et al., 2014). Less severe childhood experiences such as lower socioeconomic position in childhood but not in adulthood were also shown to associate with specific gene expression (Miller et al., 2009) and DNA methylation (Borghol et al., 2012) profiles in adult peripheral tissue. Not only blood cells but also saliva and buccal epithelial cell DNA methylation alterations have been found to associate with childhood maltreatment and depressive symptomatology in children (Weder et al., 2014), parental stress in adolescents (Essex et al., 2011) and victimization/bullying in monozygotic but discordant twins (Ouellet-Morin et al., 2013).

Although different signatures in response to early life events are observed in different tissues, epigenetic changes in peripheral tissues may correlate to some extent with measures in the brain. While in healthy controls, between-tissue variation in DNA methylation was indeed found to greatly exceed between-individual differences within any one tissue, Davies et al. identified selected inter-individual variations that were indeed reflected across both brain and blood samples (Davies et al., 2012). Furthermore, differential DNA methylation of the *serotonin transporter* (*SLC6A4*) gene promoter in T cells and monocytes seen with differences in childhood limited physical aggression in men was also found associated with in vivo measures of human brain serotonin synthesis (Wang et al., 2012). Together, these data indicate that peripheral tissues could be useful in clinical and epidemiological studies of complex neurobiological phenotypes.

The observations that ELS-associated DNA methylation changes are not limited to the brain but are also observed in peripheral systems highlight that such changes may be induced by system-wide influences. The long-lasting effects of ELS are thus likely also reflected in tissues outside the brain with additional health risks, such as the described increased risk for cardiovascular and metabolic diseases (Danese and McEwen, 2012). In rhesus macaques, the broad impact of maternal rearing in the first year of life on DNA methylation was seen in both the brain and T cells, supporting the hypothesis that the response to early-life adversity is system-wide and genome-wide and persists to adulthood (Provencal et al., 2012). As suspected, this response to early adversity is not limited to DNA methylation but other epigenetic mechanisms are also involved. For example, DNA hydroxymethylation in the prefrontal cortex (PFC) of these differentially reared monkeys was altered in specific genes involved in neuronal functions that did not show altered DNA methylation patterns (Massart et al., 2014).

Together these studies indicate that there is an epigenetic component in the biological embedding of postnatal stress shaping risk and resilience for the subsequent development of behavioral and psychiatric disorders. Part of these epigenetic alterations is also observed in peripheral tissues, such as blood. ELS-associated changes in organs other than the brain may be causally linked to the increase in risk for cardiovascular and metabolic disorders (Danese and McEwen, 2012). An increasing number of studies have now documented that ELS-exposure leads to proinflammatory immune profiles in adulthood (Danese et al., 2007) as risk factor for cardiovascular disease and increases in body mass index, and likely associate with increases in metabolic risk profiles (Danese and Tan, 2014). The question, however, of which mechanisms link environmental exposure to epigenetic changes remains mostly unanswered. In the following sections, we attempt to summarize and critically review the evidence for the possible molecular pathways involved in ELS-associated changes in DNA methylation.

Possible mechanisms involved in the epigenetic alterations following postnatal stress

The most cited paper in relation to epigenetic programming in response to early life stress is a study performed by Ian Weaver, Moshe Szyf and Michael Meaney and colleagues which has shown that DNA methylation patterns of the *Nr3c1* gene can be altered after birth in response to social experience. The authors studied the impact of maternal care on epigenetic programming of the *Nr3c1* gene promoter in the rat hippocampus (Weaver et al., 2004a, 2004b). The mRNA expression of the *Nr3c1* gene was shown to be decreased in the hippocampus of adult rat offspring of mothers that displayed decreased levels of pup licking and grooming (LG) over their first week of life, interpreted as a decreased or at least altered level of maternal care. These pups also exhibited higher behavioral and endocrine responses to stress in adulthood. This differential expression between low LG and high LG offspring was associated with a difference in DNA methylation and histone acetylation in the rat *Nr3c1* exon 1_7 (human exon 1_F) in the hippocampus. While these differences were maintained until adulthood in the untreated rat, they could be prevented by cross-fostering experiments as well as reversed by central infusion of a histone deacetylase inhibitor in adult rats. These treatments abolished the group differences in histone acetylation, DNA methylation, *Nr3c1* expression and the endocrine and behavioral responses to stress in adulthood. The study also demonstrated for the first time that DNA methylation changes in response to the environment are triggered by a molecular signaling cascade. The DNA methylation difference was located within the *Nr3c1*'s promoter nerve growth factor inducible A (NGFI-A) transcription factor binding site that is responsible for proper *Nr3c1* transcription. Exposure to high LG increases hippocampal serotonin that activates NGFI-A through a serotonin receptor signaling pathway that induced cyclic adenosine monophosphate (cAMP) (Weaver et al., 2007). Activated NGFI-A then recruits the histone acetyltransferase CREB binding protein (CBP) to the DNA and this in turn triggers DNA demethylation. These results illustrate how an environmental stimulus, touch from licking and grooming of the mother, can lead to epigenetic programming that influences life-long behavioral trajectories.

Another important study using early life maternal separation also suggests that these long-term changes in gene expression and DNA methylation are triggered by neuronal activity (Murgatroyd et al., 2009). The authors report that early postnatal maternal deprivation (PND1-10) in mice increases *Avp* mRNA expression and decreases

DNA methylation in the *Avp* gene enhancer region in the paraventricular nucleus (PVN) of 6 weeks, 3 months and 1-year-old mice. AVP is a central peptide in the hypothalamic stress hormone regulation. This increase in *Avp* expression was mediated by an increase in phosphorylation and release of methyl CpG-binding protein 2 (MeCP2) binding from the *Avp* enhancer in 10 days old mice but without accompanying changes in DNA methylation at this age. MeCP2 is a transcription regulator expressed in all tissues, especially in neurons where it is involved in neuronal development as well as in the control of the stress response. It binds to methylated DNA and recruits other chromatin modifying enzymes and, in this case, brings about gene silencing. The authors further show that this increase in MeCP2 phosphorylation at 10 days was mediated by neuronal activation (depolarisation) (Chen et al., 2003; Martinowich et al., 2003) and increased activation of CA^{2+}/calmodulin-dependent protein kinase II (CaMKII), a kinase previously reported to phosphorylate MeCP2 (Zhou et al., 2006). Following the phosphorylation and release of MeCP2 from the *Avp* enhancer region by ELS at PND10, DNA demethylation of the *Avp* enhancer is observed at 6 weeks of age and actively maintained throughout the life span. This study not only highlights an important mechanism involved in biological embedding of ELS but also emphasizes the relevance of sequential establishment of these altered epigenetic marks for long-lasting behavioral effects. Recent evidence from the same group suggests that this early priming to demethylation by ELS exposure could be preceded by the binding of polycomb repressive complexes and TET proteins to the locus (Murgatroyd and Spengler, 2014). In cell culture experiments, they could show that polycomb associated with TET proteins are released from the *Avp* enhancer during hippocampal cell differentiation. This then leads to DNA methylation and recruitment of MeCP2 together with DNMT and HDAC to maintain *Avp* repression. This control over *Avp* expression could then be released by ELS-dependent phosphorylation and dissociation of MeCP2 as shown in their first paper. Here, the establishment of *Avp* enhancer methylation enables programming of *Avp* expression in response to ELS. This fits very well with a larger body of evidence linking TET proteins with neuronal activity-dependent DNA demethylation observed in fear conditioning and memory formation paradigms (Guo et al., 2011a, 2011b; Li et al., 2013; Sultan and Sweatt, 2013).

While animal experiments of ELS mostly use genetically identical animals, environmental exposure in humans falls on a genetically diverse background, leading to large differences in the long-term behavioral/psychiatric outcomes (Kendler et al., 1999; Sartor et al., 2012). There are now a number of examples for gene x environment interactions (GxE) where a genetic polymorphism moderates the outcome to exposure to stressful life events (Bennett et al., 2002; Caspi et al., 2002; Klengel et al., 2013). One of these examples is a SNP located in intron 2 of the *FK506 binding protein 5* (*FKBP5*) gene. FKBP5 regulates the sensitivity of GR, in that increased FKBP5 levels are associated with GR resistance and a prolonged stress response by impairing negative feedback. In addition, *FKBP5* expression is induced following stress exposure through the binding of the GR to glucocorticoid responsive elements (GRE) located in the regulatory regions of the gene (Makkonen et al., 2009), thus providing an ultra-short feedback loop for GR sensitivity. The genetic polymorphism conveying GxE was found to influence the three dimensional loop formation between a GRE in intron 2 and the transcription start site (TSS) thereby changing the transcriptional activation of *FKBP5* following stress (Klengel et al., 2013). This intronic SNP in *FKBP5* not only alters the molecular response to stress, but also the systemic response by leading to prolonged cortisol release after stress (Buchmann et al., 2014; Ising et al., 2008) and altered amygdala and hippocampal activation in threat related tasks (Fani et al., 2013; Holz et al., in press; White et al., 2012). Importantly, this SNP was not only shown to moderate primary physiological responses to stress but also long-term risk for a number of psychiatric disorders, including PTSD, depression, psychosis, aggression and drug abuse (Appel et al., 2011; Bevilacqua et al., 2012; Binder et al., 2008; Collip et al., 2013; Klengel et al., 2013; Zimmermann et al., 2011) following early trauma exposure. With exposure to trauma in childhood, the initial hyper-induction of *FKBP5* by the genetic predisposition was shown to be followed by glucocorticoid-induced DNA demethylation of another enhancer element in intron 7 of the gene (Klengel et al., 2013). Interestingly, this demethylation following exposure to early trauma was only seen in carriers of the risk alleles that are also the *FKBP5* high-induction alleles. Other studies have reported similar results where the DNA sequence itself encodes information on its methylation status in *cis* (local genotype that is associated with DNA methylation on the same DNA molecule) (Gertz et al., 2011; Gibbs et al., 2010; Kerkel et al., 2008; Meaburn et al., 2010; Zhang et al., 2010a, 2010b, 2010c). Environmentally-induced epigenetic changes may thus have sequence specific effects where individual who carry the genetic predisposition are more sensitive to long-lasting epigenetic alterations following ELS and therefore are at higher risk to develop psychiatric and behavioral disorders.

In summary: The above studies indicate that early life environment can have an impact on DNA methylation in the brain that is likely mediated through specific signaling pathways following neuronal activation. On the other hand, epigenetic effects of glucocorticoids might have more widespread epigenetic effects, possibly affecting a number of target tissues. GR induced local active DNA demethylation has initially been described in liver cells (Thomassin et al., 2001) and likely involves base-excision repair. We could show that the early trauma associated demethylation in the *FKBP5* intronic GREs observed in DNA from peripheral blood was also seen in a neuronal hippocampal progenitor cell line following exposure to GR agonists (Klengel et al., 2013) suggesting similar epigenetic mechanisms in both blood and brain tissues. These common effects may explain why the interaction of *FKBP5* risk variants and ELS increases the risk not only for psychiatric disorders but also negative physical health outcomes (Zannas and Binder, 2014).

Stress in utero

Increases in anxiety or depression-like behaviors have been observed in the offspring of mothers exposed to stress during pregnancy in a number of different species, including rodents (de Souza et al., 2013; Lee et al., 2007; Markham and Koenig, 2011; Rayen et al., 2011; Vallee et al., 1997), primates (Schneider et al., 2002) and humans (Huizink et al., 2003) suggesting common cross-species mechanisms. Maternal intake of steroid hormones (Marciniak et al., 2011a, 2011b), maternal depression (O'Connor et al., 2014) and maternal anxiety (Kane et al., 2014) were all shown to alter maternal glucocorticoid levels which is hypothesized to also alter fetal exposure to glucocorticoids. While antenatal glucocorticoids are critical for the proper maturation of vital organs and tissues during pregnancy, especially in the third trimester (Whitelaw and Thoresen, 2000), pathologically increased levels of fetal glucocorticoids are suspected to impair normal development with long-term adverse consequences such as dysregulation of the infant's HPA axis and neurobehavioral changes (Lester and Padbury, 2009; Li et al., 2012; Marciniak et al., 2011a, 2011b; Sarkar et al., 2001).

A central question arising from these studies is how increases in cortisol levels in the mother can affect fetal development? One mechanism that has been proposed is an alteration in placental permeability that leads to an impaired protection of the fetus from maternal insults. This is supported by the high correlation ($r = 0.62$, $p < 0.001$) between maternal and fetal plasma cortisol in healthy pregnant mothers (Gitau et al., 1998), despite the much lower (over 13 fold) levels in the fetus. This suggests that an increase in maternal cortisol could lead to an increase in fetal levels and this could be further increased through possible deficit in placental regulation. The prime target for alterations in placental cortisol permeability is 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), as this enzyme converts excess cortisol to inactive

cortisone. In rats, maternal stress during pregnancy has been shown to decrease placental 11β-HSD2 mRNA levels and activity (Lucassen et al., 2009; Mairesse et al., 2007), thus impairing the conversion of maternal glucocorticoids to less active forms. Such stress-induced changes in placental permeability may be mediated and accompanied by epigenetic changes in a number of target genes including *11β-HSD2* (Banister et al., 2011; Lester and Padbury, 2009). Examples for such placental epigenetic regulation through maternal stress have been reported and are described in the next section.

Evidences for epigenetic alterations following stress in utero

Twin studies using monozygotic (MZ) as well as dizygotic (DZ) twins indicate that both intrauterine environmental exposures and genetic factors contribute to the establishment of the neonatal epigenome (Ollikainen et al., 2010). This highlights the fact that the intrauterine period is a sensitive time for the establishment of epigenetic variability in humans and therefore adverse events during this critical period may induce dramatic changes on the neonate epigenome. Here, we will describe some of the evidence that epigenetic alterations in the placenta of critical genes involved in the HPA axis are associated with stress in utero and can contribute to long-term developmental differences and long-term effect on health in the offspring.

In the placenta, GR is expressed at high levels as well as many neuropeptide hormones that are analogous to those produced by the hypothalamus and pituitary of the brain, including GnRH, TRH, GHRH, CRH, and oxytocin (Liu et al., 2009). A recent study, investigated the DNA methylation levels of candidate genes involved in cortisol signaling and bioavailability in placenta of early- and late-onset preeclampsia (Hogg et al., 2013). Glucocorticoids have been shown to be elevated in fetal blood in intrauterine growth retardation and preeclampsia as well as higher placental cortisol levels are observed in preeclampsia. The authors observed a significant increase in methylation at the *NR3C1* exon 1D promoter and *CRH binding protein* intron 3 in the placenta with both early- and late-onset preeclampsia. In contrast, no changes in DNA methylation in the *11β-HSD2* gene promoter were observed. Altered methylation and expression of the *NR3C1* exon 1F transcript in the placenta was also shown to associate with differences in uterine growth (Filiberto et al., 2011). These differences in *NR3C1* gene methylation were also correlated with infants' quality of movement as well as attention (Bromer et al., 2013). These changes in DNA methylation are believed to alter placental gene expression and by this placental permeability leading to altered fetal exposure to cortisol. One study examining the possible mechanisms how such epigenetic changes alter placental permeability to cortisol is described in the next section.

Moreover, a recent study by Teh et al. identified 1435 loci in the methylome of neonates to be highly variable across individuals. Interestingly, most of these variable regions were best explained by the interaction between the genotype and different in utero environments, including maternal smoking, maternal prepartum depression, maternal BMI, infant birth weight, gestational age and birth order (Teh et al., 2014). Together these data suggest that alterations of the uterine environment might induce long-term changes on the offspring's epigenome including in genes involved in the stress response which could influence normal development and lead to a dysregulation of the infant's HPA axis.

Possible mechanisms involved in the epigenetic alterations induce by stress in utero

The first evidence that prenatal environmental exposures may indeed alter the adult phenotype through epigenetic remodeling came from a mouse study. In a classic experiment, the lab of Jirtle demonstrated that maternal methyl-rich diet during pregnancy can change the offspring's coat color and growth phenotype in agouti mice, and that this was mediated by DNA methylation of a transposable element (Waterland et al. 2003). Exposure of a pregnant mother during gestation to methyl-rich diet increases the availability of methyl groups in the offspring and therefore increases the DNA methylation capacity for DNA methyltransferases.

As described above, a key enzyme controlling the placental transfer of maternal glucocorticoids to the fetus is 11β-HSD2. Placental 11β-HSD2 mRNA levels were shown to decrease following chronic restrain stress during gestation in rats and this was accompanied with an increase in DNA methylation at 3 CpG sites in the promoter of the *11β-HSD2* gene (Jensen Pena et al., 2012). In addition, Jensen Pena et al. also found an increase in placental mRNA levels of DNMT3a, an enzyme responsible for de novo methylation, following chronic stress. An increase in this enzyme may contribute to the observed hypermethylation of the *11β-HSD2* gene promoter. These findings suggest that maternal stress might alter the permeability of the placenta to maternal glucocorticoids by altering the epigenetic regulation of placental *11β-HSD2*, and by this further increasing the corticosterone levels in the fetus. However, corticosterone levels were not accessed in this study. In addition, changes in *11β-HSD2* methylation were not only observed in the placenta but also in the fetus, with increased promoter and decreased exon 1 methylation levels in the *11β-HSD2* gene in the hypothalamus following exposure to prenatal stress. No change in *11β-HSD2* DNA methylation was observed in the fetus cortex but significant increases in DNMT1 mRNA level were observed in this tissue. These findings also highlight the specificity and complexity of the epigenetic effects observed following chronic prenatal stress where longitudinal studies in multiple tissues are needed to delineate the mechanisms involved.

A study conducted by Howerton et al. in mice indicates that the effects of prenatal stress are sex-specific with male offspring being more sensitive than female offspring as they displayed a long-term increase in HPA axis reactivity not seen in females (Howerton et al., 2013; Mueller and Bale, 2008). Using gene expression arrays, the authors identified the O-linked-N-acetylglucosamine transferase (*OGT*) transcript to be decreased in the male vs. female placenta with a further decrease of that transcript observed following prenatal stress in both sexes. Chromatin immunoprecipitation analysis revealed a decrease in H3K4me3, a permissive chromatin mark, in the promoter region of *OGT* in male and prenatally stressed placentas. OGT, an X linked gene, plays an important role in the regulation of chromatin remodeling proteins such as histones. OGT-dependent histone O-GlcNAcylation leaves marks on histone 2B that are associated with gene transcription regulation (Chen et al., 2013). Moreover, female mice hemizygous for an *OGT* deletion were shown to have altered hypothalamic gene expression and broad microRNA changes when compared to wild-type females. In addition, OGT was recently demonstrated to interact with TET2, an enzyme responsible for the conversion of 5-methylcytosine into 5-methylhydroxycytosine. Their interaction was observed at transcription start sites and facilitates the establishment of OGT-dependent histone O-GlcNAcylation marks on histone 2B. These results suggest a dual epigenetic role for OGT in neurodevelopment via chromatin remodeling of various gene loci as well as changes in microRNA profiles. Together, these data suggest that prenatal stress might affect multiple genes and microRNAs through its epigenetic alteration of *OGT* expression and O-GlcNAcylation profiles. The sex-specific effect on the phenotype may be explained by the fact that this X-linked gene is less expressed in males to begin with.

Together, this concatenation of data indicates that stress during pregnancy affects the expression of a number of key genes that may alter the permeability of the placenta to glucocorticoids possibly through alteration in 11β-HSD2 function that further increases glucocorticoid levels in the fetus and leads to long-term changes in neurodevelopment. In addition, prenatal stress may also directly impact the function of enzymes involved in epigenetic regulation, such as OGT, leading to genome-wide epigenetic effects of prenatal stress and long-term effects on neurodevelopment and stress hormone regulation.

Stress pre-conception and its transmission to the following generations

Above, we have described the evidence for epigenetic mechanisms to be involved in the biological embedding of stress during early life and pregnancy. But what happens if the parents were exposed to high levels of stress during their lives? Can there be a biological embedding of this exposure in the offspring? It could be of relevance if information on the environmental conditions the parents lived in is transmitted to the offspring so that they can adequately respond to the environment they will be born into.

Epidemiological studies have reported such associations when investigating parental food supply and risk for cardiovascular diseases, obesity, type-2 diabetes, breast cancer, longevity and schizophrenia in the offspring (Kaati et al., 2002; Lumey et al., 2011; Painter et al., 2005; Susser and St Clair, 2013). For example, some of these studies focused on the Dutch famine cohort. In this cohort, transgenerational effects of maternal under-nutrition during gestation were identified in the grandchildren, where adult offspring (F2) of in utero exposed men (F1) but not of exposed women were more obese (Veenendaal et al., 2013). This sex-specific transgenerational effect of gestational famine has been previously observed in a Swedish cohort in which grandparents exposed to limited food supply influenced the grandchildren's mortality risk ratio in a sex-specific manner through the paternal lineage (Pembrey et al., 2006). In the Dutch famine cohort, the individuals exposed to prenatal famine also displayed altered DNA methylation patterns (Heijmans et al., 2008). Not only food restriction but also exposure to trauma was shown to have such transgenerational effects in humans (Kellermann, 2013). Ullmann et al. observed an increase rate of depression and psychosomatic symptoms in the third generation of Jewish grandparents exposed to the Holocaust (Ullmann et al., 2013). Exposure to child abuse and neglect of the mother was also shown to greatly increase the risk to develop behavioral abnormalities and neurodevelopmental disorders in the offspring (Dubowitz et al., 2001; Miranda et al., 2011). Comparably in rats, pups of mothers that were stressed prior to reproduction and pregnancy also display anxiety and depression-like behavior (Huang et al., 2013; Ryzhavskii et al., 2001).

These effects seen in the offspring of parents' exposed to adverse environments can be transmitted by a number of different mechanisms, including changes in the parenting behavior of the traumatized parent or changes in the in utero milieu mediated by altered placental cortisol inactivation for example. An additional explanation can be that parental stress is registered in the parent's germline and is directly transmitted to the zygote in the first stages of development. For the mother exposed to stress in her childhood or adulthood, the mature oocytes will also be exposed to the stress since all the oocytes are already formed in the ovary. In the fathers exposed to stress, spermatogonia will have to register stress and transmit this information to the mature sperm. In humans, it is impossible to disentangle all these factors and the dissection of the different contributions of these mechanisms has to happen in animal models as described below.

Mechanisms for intergenerational transmission of stress

Evidence for an additional direct transmission of parental or ancestral stress through the germ line comes from a handful of recent studies. In a mouse model, paternal chronic stress exposure induced decreased HPA axis reactivity in both male and female offspring and this was accompanied by global changes in gene expression in the PVN and bed nucleus of stria terminalis (BNST) (Rodgers et al., 2013). These global changes are consistent with altered offspring stress responsivity as an increased expression of glucocorticoid-responsive genes was observed in the PVN. The authors suspected that these global changes were due to epigenetic reprogramming. They indeed report several lines of evidence suggesting that some of these changes are transmitted via altered microRNAs content in the sperm. Significant changes in microRNAs levels in the sperm of the stressed males were observed when compared to controls. MicroRNAs have been shown to alter gene expression post-fertilization, suggesting that these changes in the sperm microRNA content could impact the offspring development (Giraldez et al., 2006; Grandjean et al., 2009; Rassoulzadegan et al., 2006). In addition, the same group found that male offspring subjected to prenatal stress displayed a more female-like brain and behavioral phenotypes than the non-exposed controls (Mueller and Bale, 2008). This effect was transmitted to the F2 generation through the paternal lineage (Morgan and Bale, 2011), suggesting again the importance of the sperm as a vehicle for transmission of transgenerational stress signals. In addition, a significant decrease in microRNAs in the brain of the male offspring in this F2 generation was observed, that resembled the pattern observed in F2 females. Taken together these results suggest that non-coding RNAs may serve as an epigenetic mechanism responsible for the transmission of environmental cues to the next generations.

In another study, Franklin et al. reported that chronic and unpredictable maternal separation and maternal stress from postnatal days 1 to 14, leads to depressive-like behaviors and alters the response to novel and aversive environments in the offspring. They observed lower DNA methylation levels in a CpG island located around the TSS of the *corticotropin releasing hormone receptor 2* (*Crhr2*) gene in the sperm of the male offspring that were raised by stressed mothers compared to controls (Franklin et al., 2010). *Crhr2* was previously shown to be involved in depressive-like behavior (Weiser et al., 2008). A comparable decrease in DNA methylation at many CpG sites in the *Crhr2* locus was also observed in the brain of animals of the following generation. The DNA methylation changes in the sperm were small (<5%), suggesting that these small effects in the sperm may indeed have an important role in the regulation of this gene in the offspring. The authors also identified a CpG island in the promoter of the *MeCP2* gene to be hypermethylated in the sperm of the males offspring exposed to chronic maternal stress. Similar to the *Crhr2* results describe above, the difference in methylation observed was relatively small (<5%) but this difference increased to ~10% in the brains of the animals of the following generation. These data indicate that chronic maternal stress is transmitted to the following generation through the male germ cells and that epigenetic mechanisms in the sperm and mature tissues of the offspring might be part of this intergenerational effect. This theory could be recently expanded to include a role for the sperm microRNAs. Exposure to postnatal stress was associated with specific changes in microRNA patterns in the sperm as well as in multiple other tissues of the F1 generation and also the F2 generation (Gapp et al., 2014). A direct evidence for the importance of these sperm microRNAs was provided by injecting the sperm RNA of males subjected to unpredicted maternal stress and separation into wild type fertilized oocytes. Comparable behavioral, metabolic and molecular effects were seen in the offspring from these manipulated fertilized oocytes than in offspring of father exposed to stressors in early postnatal life. These data strongly suggest an involvement of the altered sperm microRNA profiles by early postnatal stress in the transgenerational transmission of these effects.

Another publication by Dias and Ressler also illustrates this concept (Dias and Ressler, 2014). Here, adult mice (F0) were subjected to fear conditioning to a specific odor prior to conception. This led to an increase behavioral sensitivity to this odor, but not other odors as well as odorant receptor-specific neuroanatomical alterations in both sexes of the following generation (F1). This transgenerational transmission of fear memory to a specific odorant was conserved after cross-fostering and in vitro fertilization, again suggesting a mode of inheritance through the gametes. In fact, DNA methylation levels in the olfactory receptor gene responsible for the detection of the specific odor (acetophenone), the *Olfr151* gene locus, were shown to be altered in the sperm. This significant decrease in DNA methylation (~10 to 20%) seen in the F0 generation persisted in the F1 generation. These data suggest that even very specific environmental information, such as the association of a specific odor with adverse experiences may be transmitted to the next generation via epigenetic changes in the sperm.

These novel studies using cross fostering, in vitro fertilization and direct manipulation of the fertilized oocyte suggest a true transmission through the gametes as they can exclude effects of parental behavior and effects due to altered in utero environment.

Conclusions

There is increasing evidence for a prominent role of epigenetic mechanisms in embedding long-term effect of stress at different developmental stages as well as across generations. These epigenetic mechanisms are distinct for the different stages of stress exposure. Neural activity and stress hormone receptor induced epigenetic changes have been observed with postnatal stress exposure, leading to long-term changes not only in the brain, but also in peripheral tissues. These more global effects could explain some of the adverse effects of ELS on immune, cardiovascular and metabolic systems. Maternal stress either during or prior to pregnancy can also have a long-term impact on the offspring by changing the uterine milieu. Here epigenetic effects on key enzymes and genes in the placenta seem to play a critical role by altering the placental permeability to glucocorticoids or by direct effects on genes involved in downstream epigenetic effects. Finally, exposure to stress can also affect the next generations and these effects are directly transmitted by the gametes, with the strongest evidence for the involvement of stress-regulated microRNAs in the sperm. Exposure to stress early in life or prior to birth of an individual may prime or poise the system through epigenetic differences to future response to environmental challenges. These effects may be silent until the system is challenged in a specific way. If the environmental conditions of an individual or his descendants are different, these epigenetic memories might never express themselves or lead to maladaptive responses. The consequences of these priming effects will thus depend on the subsequent environmental exposures and may be beneficial as well as detrimental, depending on the challenge. A full understanding of these effects will require longitudinal studies starting before conception with repeated sampling of different tissues, outcomes and environmental exposure. In fact, some effects may be beneficial for the response of a specific organ but not another. In addition, genetic variation likely alters these long-term epigenetic effects of such environmental exposure, so that a combined investigation of genetic, environmental and epigenetic factors in such longitudinal cohorts is warranted.

Ultimately, a deeper understanding of these mechanisms may allow early detection of risk and guided prevention and intervention strategies to avoid the long-term negative effects of such exposures.

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