Looking beyond the DNA sequence: the relevance of DNA methylation processes for the stress–diathesis model of depression

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The functioning of the hypothalamic–pituitary–adrenal (HPA) axis and serotonergic (5-HT) system are known to be intertwined with mood. Alterations in these systems are often associated with depression. However, neither are sufficient to cause depression in and of themselves. It is now becoming increasingly clear that the environment plays a crucial role, particularly, the perinatal environment. In this review, we posit that early environmental stress triggers a series of epigenetic mechanisms that adapt the genome and programme the HPA axis and 5-HT system for survival in a harsh environment. We focus on DNA methylation as it is the most stable epigenetic mark. Given that DNA methylation patterns are in large part set within the perinatal period, long-term gene expression programming by DNA methylation is especially vulnerable to environmental insults during this period. We discuss specific examples of genes in the 5-HT system (serotonin transporter) and HPA axis (glucocorticoid receptor and arginine vasopressin enhancer) whose DNA methylation state is associated with early life experience and may potentially lead to depression vulnerability. We conclude with a discussion on the relevance of studying epigenetic mechanisms in peripheral tissue as a proxy for those occurring in the human brain and suggest avenues for future research.

1. Introduction

Two of the most convincing biological correlates of mood disorders are lower serotonin (5-HT) function [1,2] and altered hypothalamic–pituitary–adrenal (HPA) axis activity [3–5]. Indeed, alterations in both systems have consistently been associated with mental health in general and with mood disorders in particular [2]. Lower 5-HT has been associated with altered physiological functions, including cardiac function and HPA axis regulation as well as aggressive and impulsive behaviours, sleep, feeding behaviours, cognitive function and mood [2,6–10]. Alterations in the 5-HT system in mental illness occur at multiple levels through synthesis, receptor function, transport, reuptake and degradation [11]. However, the underlying physiology of altered 5-HT neurotransmission has yet to be resolved. Likewise, (dys)regulation of the HPA axis activity has also been widely implicated in depression [12,13], as

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demonstrated by an altered response to the dexamethasone/corticotropin-releasing hormone (CRH) challenge in some depressed patients [13]. Again, altered functioning of the HPA axis could occur at different levels, through hypo- or hyper-activation as well as through receptor activity.

Here, we posit that changes in serotonergic and HPA activity could stem from epigenetic changes following environmental stress. The overarching hypothesis is that environmental stressors reprogramme both the regulation of the 5-HT system and the HPA axis through epigenetic processes by altering the expression of genes involved in the function of these two systems. These epigenetic changes are not stochastic but may in fact reflect a global response to an environmental trigger. This provides an explanation for how gene and environment physiologically interact in the development of (risk for) depression.

2. Serotonergic system

Although the earliest 5-HT theories of depression considered altered 5-HT as its direct cause, current theories postulate that alterations in 5-HT are a biological risk factor for the development of depression in the presence of adversity. This so-called diathesis–stress model has been the most widely used neurobiological model of depression over the past two decades [2,8]. A number of 5-HT polymorphic genes that may increase the risk to develop depression were tentatively identified and investigated. Unfortunately, unlike important breakthroughs made in the discovery of gene variants for Mendelian disorders, identification of candidate genes for depression has shown little progress. No candidate gene has been unequivocally associated with increased risk for depression. To date, results of genome-wide association studies are inconclusive. A number of large-scale epidemiological longitudinal studies have tested possible interactions of candidate polymorphisms in 5-HT regulatory genes. These candidate genes were chosen because they have been tentatively associated with risk of depression and with environmental adversity (gene by environment (G × E) interactions). Although these models are highly plausible from a clinical perspective, empirical evidence supporting these models is limited. This is well illustrated by the example of the widely studied 5-HT transporter gene (SLC6A4, also known as 5-HTT or SERT).

(a) Structure and function of SLC6A4

SLC6A4 is widely distributed in both central and peripheral systems. In the brain, SLC6A4 can be found in the neurons containing 5-HT neurotransmitters, such as the median and dorsal raphe nuclei, cerebral cortex and the CA1 and CA3 regions of the hippocampus [14]. Peripherally, SLC6A4 is expressed in the gastrointestinal tract, lungs, placenta, blood platelets and lymphocytes [14–16]. The major function of SLC6A4 is the reuptake of 5-HT from extracellular space. The removal of 5-HT from the synaptic cleft is important for the modulation of the strength and duration of 5-HT-mediated neurotransmission. SLC6A4 also governs the recycling of 5-HT and affects the function of the 5-HT system.

(b) SLC6A4 and integrity of the brain

In humans, molecular imaging studies have suggested that alterations in the SLC6A4 gene could impact the development and function of brain regions involved in emotion regulation, including the amygdala, anterior cingulate and other prefrontal cortical areas. Consistent with the 5-HT alterations and various subtle neurodevelopmental alterations observed in SLC6A4 knockout mice [34–36], carriers of the s allele showed amygdala hyper-reactivity [37–51] upon exposure to emotional stimuli as well as reduced functional connectivity between the amygdala and the perigenual cingulate cortex [37]. They
also showed decreased grey matter volume in limbic structures (e.g. anterior cingulate, amygdala, hippocampus, perigenual cingulate cortex) [37,52,53].

Given the important role of SLC6A4 in development [34–36], psychosocial stressors in early life significantly alter 5-HTT function, and much work shows that this alteration is stable throughout life [54]. For instance, rhesus macaques that experienced high rates of rejection from their mothers soon after birth have lower levels of 5-HTT binding which is independent of 5-HTT genotype [55]. Another study compared SLC6A4 density in adulthood as a function of common natural variation in birth weight in rats [56]. It was found that rats which were 10–20% lighter at birth than their littermate had reduced 5-HTT density in the frontal cortex in adulthood, while there were no differences in noradrenaline transporter density. This suggests that normal variation in birth weight may account for subtle variations in 5-HT function later in life. This study is particularly relevant, as birth weight has been shown to be one of the best markers of in utero adversity and a predictor of developmental behavioural, physiological and cognitive outcome in humans [57–65]. The role of SLC6A4 in emotion (dys)regulation and brain development, in combination with its sensitivity to environmental changes, makes it a promising molecular mechanism accounting for G × E interactions in depression.

3. Hypothalamic—pituitary—adrenal axis

In synergy with the sympatho-adrenomedullary axis, the HPA axis is a crucial player in determining stress reactivity. In response to an external or internal stressor, there is release of the CRH and arginine vasopressin (AVP) from the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus. This release in turn triggers release of the adrenocorticotropic hormone (ACTH) from the pituitary gland into the blood. Glucocorticoids (GCs) are then produced and released by the adrenal cortex (i.e. corticosterone in the rat; cortisol in primates) [66]. GCs bind to two types of receptors: the glucocorticoid receptors (GRs) found throughout the brain and the mineralocorticoid receptors (MRs) found mainly in the limbic system, particularly in the hippocampus [67,68]. Under basal conditions, the MRs are mainly occupied owing to their higher affinity compared with GRs [68]. However, when levels of GCs increase during stress, higher occupancy of the lower affinity GCs can be observed, and a signal is sent to decrease HPA activity to restore homeostasis [68].

The stress response is adaptive and beneficial in the short-term, but when excessively activated, may lead to altered responses to stress in later life, either through down- or upregulation. Most often, repeated early stress is followed by increased basal HPA activity as well as an increased HPA response to acute stressors [68,69]. This alteration in the stress response could be achieved through action of GCs on their receptors. Once GCs are released, they bind to their receptors, which act as ‘ligand inducible transcription factors’ [4] that can activate or repress gene transcription and thus alter gene expression (reviewed in [4,70]).

(a) Glucocorticoids during development

Glucocorticoids are necessary for proper foetal development owing to their critical roles in brain and lung maturation [71–73]. These developmental effects of GCs are facilitated through binding to GRs and MRs that lead to gene expression changes. Though sources of GCs include both the mother and foetus, maternal GC levels are much higher than in the foetus. The foetus is protected from maternal GCs by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD2). HSD2 is broadly expressed in the brain and placenta to regulate foetal development. HSD2 readily converts the active GCs to the inert forms to prevent excessive activation of MRs and/or GRs [3,74]. Although this barrier only allows 10–20% of maternal GCs to access the placenta, heterogeneity exists and the amount of GCs reaching the foetus has an effect on birth weight [3]. In humans, during late gestation, around weeks 19–26, levels of HSD2 drop to allow the proper cell differentiation required for foetal development [3,74].

Repeated prenatal stress, producing chronic excessive GC activity, could potentially alter brain development and set off lifelong alterations in the HPA axis. In support of this, prenatal dexamethasone treatment increases levels of corticosterone and ACTH in offspring, impacting on the development of CRH neurons in the PVN and leading to mild chronic HPA hyperactivity [3]. Since GRs are present early during development while MRs appear during later gestation [3], repeated stress during programming would expose the foetus to an excess of GCs through their action on GRs. Maternal stress during pregnancy is also associated with decreased expression of HSD2, therefore exposing the foetus to even more GCs [3,74]. In rodent models, HSD2 deficiency or inhibition has been associated with alterations in the duration of pregnancy and birth weight of the offspring, although no changes are seen in the function of the HPA axis [3,74]. Presumably, these changes occur in order to prepare the organism for a harsh external environment [3,75]. The problem however stems from the discrepancy between the in utero and external environments according to the mismatch hypothesis [75]. An organism exposed to excess stress in utero is thereby maladapted to a ‘normal’ postnatal environment.

Accumulating evidence indicates that the period of programming extends beyond the prenatal period to the early postnatal environment. This has been shown through the experiments in Meaney’s laboratory (reviewed in [75,76]) demonstrating that early maternal care is a good moderator of in utero adversity [75]. Rat pups receiving low maternal care (licking-grooming and arched-back nursing) during the first week of life showed decreased hippocampal GR expression, lower GC feedback sensitivity, increased HPA axis activation and anxiety during adulthood, while rat pups exposed to high licking-grooming showed the reverse [77,78]. Cross-fostering experiments support the role of maternal care as opposed to genetics in the regulation of the HPA axis [76].

In non-human primates, offspring exposed to the variable foraging-demand paradigm showed increased cerebrospinal fluid (CSF) levels of CRH, which was accompanied by decreased CSF cortisol levels [70]. In humans, offspring of anxious and/or depressed mothers have altered activity of the HPA axis, alterations in frontal lobe activity and were more vulnerable to developing depression later in life [4]. Moreover, severe deprivation, neglect and abuse are associated with lower basal levels of GCs. Dietary protein restriction during pregnancy has led to a decrease in HSD2 activity and thus an increase in GC exposure for the foetus [3]. Although the direction of change varies, it is clear that HPA axis function is altered following stress. Type and
timing of stressors may play important roles in either down- or upregulating function of the HPA axis.

(b) Hypothalamic–pituitary–adrenal axis activity and integrity of the brain

Some brain areas such as the limbic regions, including the hippocampus, containing high levels of GRs are especially vulnerable to the effects of excessive HPA axis activity [4]. Indeed, several studies have found that prenatal stress affects development of the hippocampus, dentate gyrus, prefrontal cortex (PFC), amygdala, nucleus accumbens and hypothalamus [4,79,80]. Specifically, prenatal stress alters cell proliferation in the hippocampus and nucleus accumbens as well as in the amygdala to a lesser degree [79]. Maternal separation in rats increases CRH-binding sites in the PFC, amygdala, hippocampus and hypothalamus [4]. In addition, repeated unpredictable maternal separation in monkeys leads to increased CRH concentrations in CSF and alterations in the PFC, amygdala, hippocampus and dentate gyrus [4].

(c) Hypothalamic–pituitary–adrenal axis and mood disorders

Many studies have demonstrated associations between vulnerability for depression and alterations in HPA axis function that can occur at different levels. For instance, in rodents, studies have demonstrated that prenatal stress is associated with later depressive-like behaviour, although associated modifications to the HPA axis were not consistently found [3–5]. HSD2 knockout mice also showed increased anxious and depressive behaviours. Furthermore, Dekker et al. [12] reported that a polymorphism of the HSD1 gene, rs11119328, is associated with higher cortisol levels and greater vulnerability for depression. Harris & Seckl [3] suggested that it is an imbalance in MR–GR due to genetics, in combination with early stress that leads to vulnerability for affective disorders when exposed to further stress. They further suggested that prenatal stress alters functioning of the amygdala, which contains MR, GR, CRH receptors and CRH-producing cells.

In major depression, studies show alterations in HPA axis, most often an increase in activity, but only in a subgroup of patients [13,80–83]. This was demonstrated by an altered response to the dexamethasone/CRH challenge in depressed patients [13]. These functional alterations are accompanied by the expected structural neuronal alterations such as smaller hippocampal size and enlarged amygdala and adrenal glands. It appears that both the CRH and AVP systems are in overdrive in depressive states [82]. Furthermore, studies have reported an imbalance in GRs and MRs, a decrease in GR sensitivity, and an impaired ability to downregulate HPA axis activity [80]. In these depressed patients, activity of the HPA axis appears to normalize upon recovery; when it remains altered, it would indicate a greater risk of recurrence. It has also been shown that activity of the HPA axis normalizes with antidepressant use, though it is not clear whether this was due to recovery or was a function of the antidepressants. In rats, upon treatment with antidepressants, studies have found an increase in MR concentrations followed by an increase in GRs that led to a decrease in overall HPA activity. Finally, decreased GR mRNA was found in depressed patients, both during a major depressive episode and upon remission, as well as in their healthy first-degree relatives, suggestive of a trait abnormality contributing to a higher vulnerability. While familial vulnerability may be due to genetics, vulnerability following on from early stress may be due to programming of gene expression.

4. Epigenetic programming: basic mechanisms

(a) The epigenome

The genomic sequence of a multicellular organism is identical throughout the body, but cells in distinct tissues have different epigenomes that are driving distinct gene expression programmes [84]. In other words, the genome defines the potential genetic information, whereas the epigenome defines which genes of this potential repertoire are actually expressed. This regulation of gene expression without altering the sequence of the DNA is made possible by epigenetic modifications. These epigenetic patterns are generated during early development and are crucial for cellular differentiation. Indeed, during development, pluripotent stem cells undergo division and differentiate into numerous cell type which eventually become different organs and tissues with specific patterns of gene expression [85]. In addition to this innately driven sculpting of the epigenome during differentiation, changes in epigenetic modifications can also be observed later in life through a dynamic and reversible process triggered by external environmental influences [86].

The two major epigenetic modifications are chromatin modifications and DNA methylation. Chromatin is composed of DNA and nucleosomes, which are themselves composed of histone proteins. The remodelling of chromatin involves covalent modifications on the histone tails to promote an open or closed chromatin state, allowing (or not) for gene expression [87]. These covalent modifications on the N-terminal tails include acetylation, methylation, ubiquitination, phosphorylation and the less understood SUMOylation and ADP ribosylation and were proposed to make up a yet undelineated histone code that defines the state of gene expression [87]. DNA methylation is a covalent modification of the DNA molecule itself by enzymatic addition of a methyl group onto the cytosine ring residing in many instances in a cytosine-guanosine (CpG) dinucleotide. Methylation at regulatory regions of the gene such as promoter and enhancer regions promotes silencing of gene expression [86,88]. In addition, recent evidence suggests that microRNAs also play a role in epigenetic regulation, by silencing chromatin, degrading mRNA and blocking translation in order to regulate gene expression at different levels [86]. The combination of these tissue-specific epigenetic modifications defines the state of gene expression, which is necessary to maintain cellular function. These modifications might also adapt gene expression to corresponding environmental cues, serving to optimize survival of the organism. Here, we will focus on DNA methylation, as it is the most direct modification of the genomic sequence itself.

(b) DNA methylation

CpG dinucleotides are the predominant target of DNA methylation in the vertebrate genome and the process is catalysed by DNA methyltransferases (DNMTs) that transfer a methyl moiety from the methyl donor S-adenosyl methionine
to the 5‘ position on the cytosine ring. The establishment and maintenance of this post-replication biochemical process is essential for normal development of an organism, which occurs through two types of enzymatic DNA methylation processes, namely de novo and maintenance methylation. De novo methylation is responsible, during gametogenesis and early development, for the establishment of tissue-specific methylation patterns, while maintenance methylation occurs in somatic tissues and is responsible for conserving this methylation pattern through replication [89].

In mammals, the most studied DNMTs responsible for DNA methylation are DNMT1, DNMT3a and DNMT3b. DNMT1 is known as the maintenance methyltransferase as it has a higher preference for hemimethylated CpG dinucleotides [90,91]. Moreover, DNMT1 expression is high during the S phase of the cell cycle when DNA is replicated, supporting its posited role in replicating the methylation pattern onto the daughter strand from the hemimethylated DNA [92,93]. The DNMT3 family is known for its role in de novo methylation. In contrast to DNMT1, DNMT3a and DNMT3b show little preference between hemimethylated and unmethylated DNA [90]. They are highly expressed in embryonic stem cells, as opposed to somatic cells, suggesting that they play an important role in the establishment of the DNA methylation pattern during early development [94]. Moreover, since DNMT3s can perform maintenance methylation and DNMT1 has some de novo methylation activity [95], it was proposed that these enzymes may interact together in the maintenance of the methylation pattern. For instance, DNMT3a and DNMT3b de novo methylation may serve to compensate for insufficient maintenance methylation by DNMT1 [96].

(c) DNA methylation and gene expression
In mammals, methylation at regulatory regions of the gene such as promoter and enhancer regions promotes silencing of gene expression [86,88], through two mechanisms. The first mechanism involves direct interference of the methyl group with the binding of a transcription factor to recognition elements in DNA [89]. Transcription factor binding is required for gene activation and methylation at the DNA recognition sequence prevents them from binding to the genome and thus suppresses gene expression. The second mechanism is indirect in that methylation targets proteins that suppress gene expression. Mammalian methyl-CpG-binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4 are proteins that belong to a family characterized by a methyl-CpG-binding domain. All of these proteins, except for MBD3, are able to recognize and bind specifically to methylated DNA both in vitro and in vivo [97]. Studies have shown that these proteins can suppress transcriptional activity [98]. For instance, it has been shown that MeCP2 can silence gene expression specifically at methylated promoters but not at unmethylated promoters [99]. On a genome-wide scale the actions of MeCP2 are more complex, as it can function as both an activator and a repressor of transcription depending on the promoter context [100]. Furthermore, MeCP2 has been shown to interact with other proteins such as Sin3A and histone deacetylases [101]. These proteins form a corepressor complex that alters chromatin structure and regulates gene expression, representing a direct interaction between DNA methylation and chromatin modification [101,102].

Transcriptional repression can be relieved by inhibition of histone deacetylase, suggesting that chromatin modification is essential in this repression mechanism. Similarly, MBD1–4 are also known to interact and form complexes with various chromatin-modifying proteins and their transcriptional repression activity is heavily dependent on histone deacetylase enzymes [98]. However, it should be noted that the effect of DNA methylation is context-dependent and methylation of repressor sites can increase transcription.

In short, methylation at CpG sites can either impede transcriptional factors from binding to the DNA sequence or attract proteins to form corepressor complexes, in order to suppress gene expression. Moreover, evidence suggests that DNA methylation and chromatin modifications such as histone deacetylation can act in concert to regulate transcriptional activity [84]. High density of methylation or methylation of critical CpG sites could silence gene expression resulting in a loss of function, which could have a similar consequence as a loss of function by genetic mechanisms, such as mutation, deletion or rearrangement [87].

(d) Reversibility
It was proposed that DNA methylation can be reversible, suggesting that DNA methylation is dynamic [103]. Demethylation could occur by a passive loss of a methyl group by replication of new cytosines in the absence of a DNMT. There is however evidence that DNA demethylation is an active process that can take place in non-dividing neurons as well [104,105]. However, the exact mechanism of demethylation is still controversial. Some propose that it involves complex repair mechanisms where the 5-methylcytosine is hydroxylated, deaminated and removed by glycosylation and replaced with an unmethylated cytosine in a mismatch repair process [106–108]. Others believe that there are specific enzymes that carry out demethylation. Methyl-CpG-binding domain protein 2 (MBD2) is an enzyme that has demethylation activity and removes the methyl group from 5-methylcytosine through an oxidative process [109,110]. More studies are needed to further validate these findings.

(e) DNA methylation during development
The DNA methylation pattern is formed during gestation by a series of global methylation and demethylation events that are followed by cell-type-specific and gene-specific sculpting of the DNA methylation pattern through methylation–demethylation [111,112]. The DNA methylation pattern in this perinatal period, when it is shaped and fashioned, is highly vulnerable to environmental exposures. Indeed, restriction of certain dietary components, such as folic acid and vitamin B12 during gestation, is known to affect the DNA methylation pattern in foetal liver cells in sheep [113]. Similarly, a study in humans has shown that individuals who were exposed to famine in the perinatal period had, six decades later, altered DNA methylation patterns compared with their siblings [114]. Furthermore, a number of studies have shown associations between early trauma and DNA methylation levels in candidate genes, in particular with regard to the HPA axis stress-related genes and the SLC6A4 gene [115–118] (see below). These associations are of particular interest given their substantial role in brain development and emotion regulation.
(f) DNA methylation and behaviour

Recently, a number of studies have been published investigating epigenetic studies in relation to mental health problems, including depression. These studies will be discussed in the next sections. Although it is unlikely that the environment will affect a single gene or that a single gene will modulate the phenotype, our emphasis in this review is on DNA methylation in the SLC6A4 gene encoding the 5-HT transporter as well as in HPA axis stress response-related genes: notably the NR3C1 gene and Arp enhancer. These are the most widely investigated genes in relation to depression and the functionalities of their polymorphisms have been relatively well characterized. Finally, there is sufficient evidence of expression in peripheral blood cells of these genes. The latter is important as it is impossible to study methylation directly in the living human brain (see §5 for more details).

(g) Epigenetic studies of SLC6A4

In addition to the fixed DNA sequence, DNA methylation might explain how SLC6A4 gene expression is regulated and in turn results in behavioural changes. In human lymphoblast cell lines, higher SLC6A4 methylation was associated with reduced mRNA levels [119]. This observation suggested that it is possible to link SLC6A4 promoter methylation with gene expression. Similarly, a study in rhesus monkeys has found that ss carriers had higher mean methylation and lower SLC6A4 levels in peripheral blood mononuclear cells (PBMCs) [54]. Furthermore, it has been demonstrated that both complete and partial in vitro promoter methylation significantly reduced SLC6A4 expression [120].

To date, most studies have focused on the genetic aspect of depression, but a few studies have focused on DNA methylation of SLC6A4 or other genes in the context of mental health. Recent studies (published up to May 2012) have been summarized in table 1.

A few studies have now been conducted investigating methylation in the SLC6A4 promoter and its association with depressive symptoms in humans. In one study, human lymphoblast cell lines were obtained from a longitudinal cohort and methylation analysis of the CpG island within the SLC6A4 promoter was conducted [122]. A trend for increasing methylation with life history of depression was revealed and higher methylation of the SLC6A4 CpG island was associated with lower mRNA in females when compared with males [122]. More recently, another longitudinal study in adolescents has found that depressive symptoms were more commonly observed in ss allele carriers with elevated methylation in buccal cells [120]. Lastly, a third study has found that exposure to maternal depressive symptoms in the second trimester of pregnancy was associated with reduced maternal and infant SLC6A4 promoter methylation at specific CpG sites [121].

In addition to depression, studies have also found associations between SLC6A4 methylation and diagnoses that co-occur with depression, such as trauma [116,123] and unresolved responses to loss or trauma [124]. In contrast, some alcohol-dependence and posttraumatic stress disorder studies did not yield significant effects of DNA methylation [115,125]. However, it is worth noting that the latter two studies only included a small portion of the SLC6A4 promoter and investigated seven and two CpG sites, respectively.

In conclusion, there is evidence linking SLC6A4 methylation with depression and various psychiatric conditions that often co-occur with depression. Although these are pilot studies focusing on different SLC6A4 promoter regions and using different methods for methylation analysis, they have demonstrated a connection between peripheral SLC6A4 methylation and various behaviours that are controlled by the central 5-HT system. More epigenetic studies are needed to examine the possibility of using SLC6A4 methylation as a peripheral marker for vulnerability to depression.

(h) Epigenetic studies of hypothalamic–pituitary–adrenal regulation genes

As with the serotonergic system, epigenetic mechanisms appear to play an important role in altering the expression of genes involved in the regulation of the HPA axis. Most of these studies have investigated the impact of early postnatal maternal care behaviours.

A number of genes are involved in the regulation of the HPA axis. One of the most important is the gene encoding the GR, NR3C1. Variations of normal levels of maternal care in rodents during the first week of life programmes DNA methylation of the NR3C1 and HPA axis activity by proxy for life [126]. Following repeated mild stress in mice, studies have found decreased levels of NR3C1 mRNA in the hippocampus, hypothalamic PVN and pituitary, all regions implicated in regulation of the HPA [69,127], and this was accompanied by increased anxious behaviour [127]. Specifically, early stress such as low maternal care led to alterations in methylation of specific CpG residues that are often associated with decreased gene expression, notably the exon 17 promoter specific to the hippocampus [3,76,82,127] and decreased histone acetylation [127]. High levels of maternal care are associated with lower levels of methylation in the 5’ CpG site of the transcriptional activator of nerve growth factor-inducible protein A (NGFI-A) binding site of the NR3C1 exon 17 promoter that resulted in increased NGFI-A binding to the 17 promoter compared with low maternal care. To demonstrate that this was not a genetic effect, Weaver et al. [127] showed that methylation of the NR3C1 promoter in low-care offspring could be reversed by cross-fostering, which was accompanied by a decreased HPA response to stress. Furthermore, methylation attracts methylated DNA-binding proteins and histone deacetylases (HDACs) to prevent histone acetylation and transcription factor binding. The HDAC inhibitor trichostatin A (TSA) has been shown to play a role in replication-independent demethylation by increasing histone acetylation and chromatin activation [128,129]. If adult rats exposed to stress during infancy are treated with the HDAC inhibitor, TSA, the increased methylation and heightened HPA axis response to stress are both normalized [126], supporting the importance of the methylation process. Interestingly, recent work has suggested that maternal care may affect hippocampal NR3C1 expression by activating 5-HT receptors through cAMP and PKA. In cultured rat hippocampal neurons, 5-HT increases both NR3C1 and NGFI-A expression [76].

In human studies, early life adversity and parental care have also been shown to impact DNA methylation of genes involved in the HPA axis. Maternal prenatal depressed mood has been shown to predict NR3C1 promoter methylation in lymphocytes obtained from newborns [75]. Likewise, increased
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<tr>
<td>depression</td>
<td>[121]</td>
<td>human blood leukocytes</td>
<td>82 pregnant women</td>
<td>pyrosequencing</td>
<td>lower methylation was associated with higher depression at 26 weeks gestation in mother and infants at specific CpG sites</td>
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<td></td>
<td>[122]</td>
<td>human lymphoblast cell lines</td>
<td>96 males and 96 females</td>
<td>mass spectrometry</td>
<td>a trend of increased SLC6A4 methylation with lifetime history of depression. Higher methylation and lower mRNA observed in females than males</td>
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<td>child abuse</td>
<td>[123]</td>
<td>human lymphoblast cell lines</td>
<td>96 males and 96 females from the IOWA adoption study</td>
<td>mass spectrometry</td>
<td>higher overall methylation levels associated with history of child abuse</td>
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<td></td>
<td>[116]</td>
<td>human lymphoblast cell lines</td>
<td>155 females randomly selected from the IOWA adoption study</td>
<td>mass spectrometry</td>
<td>positive correlations between (i) childhood sex abuse and promoter methylation and (ii) antisocial personality symptoms and methylation were observed in females</td>
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<td>rearing and stress behaviour</td>
<td>[54]</td>
<td>monkey blood PBMCs (Macaca radiata) and their mothers</td>
<td>10 female bonnet macaques from the IOWA adoption study</td>
<td>mass spectrometry</td>
<td>no correlation between rearing and behaviour and methylation, but ss rs5-HTTLPR allele has higher mean DNA methylation and lower SLC6A4 expression</td>
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<td>unresolved loss or trauma</td>
<td>[124]</td>
<td>human lymphoblast cell lines</td>
<td>143 adopted participants, 50% females</td>
<td>mass spectrometry</td>
<td>higher methylation was associated with higher risk of unresolved responses to loss/trauma in s variant. Inverse correlation between methylation and risk was found in ss genotype</td>
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<tr>
<td>alcohol dependence</td>
<td>[125]</td>
<td>human blood plasma</td>
<td>27 male pts versus 15 controls</td>
<td>pyrosequencing</td>
<td>no significant methylation differences</td>
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</table>

**Table 1.** Human epigenetic studies of SLC6A4.
NR3C1 promoter methylation at the transcription factor NGFI-A-binding region was found in infants with depressed mothers and their stress response was altered with enhanced salivary cortisol level when measured at three months of age [130]. Life history of childhood abuse is also known to alter the stress response of the HPA axis and individuals who experienced childhood abuse are more vulnerable to depression and committing suicide [131–133]. In post-mortem brain samples from suicide victims, enhanced DNA methylation in the NR3C1 exon 1F promoter (the human homologue of the exon 1F promoter) and decreased NR3C1 mRNA expression were found in the hippocampus in those with a history of childhood abuse [134]. A very recent study extended these findings, showing also differential hippocampal NR3C1 expression of the 1A, 1C and 1D variants in this sample, while no group differences were observed in the anterior cingulate [135]. Interestingly, childhood abuse was also associated with differential DNA methylation and gene expression of ribosomal RNA in the brain, suggesting that it has a broad genetic effect [136]. Moreover, Alt et al. [137] found decreased NR3C1 expression in the amygdala, cingulate gyrus and inferior PFC as well as decreased NGFI-A expression in the post-mortem hippocampus of depressed patients compared with controls. Human studies of NR3C1 methylation (published up to May 2012) are summarized in table 2.

Maternal care during infancy is also associated with altered gene expression and DNA methylation of the arginine vasopressin (Avp) gene in mice. Three hours of maternal separation daily during the first 10 days of life in mice has been linked with lifelong alterations of the HPA axis accompanied by hypersecretion of corticosterone and persistent upregulation of AVP expression in the PVN of the hypothalamus [69]. This sustained AVP expression was associated with long-lasting hypomethylation at a downstream Avp enhancer in the PVN, a region that contains high-affinity context-dependent DNA-binding sites for MeCP2 [69]. Interestingly, MeCP2 binding to the Avp enhancer in the stressed mice was also greatly reduced compared with the controls. Therefore, it was proposed that early life stress such as maternal separation leads to reduced DNA methylation and MeCP2 binding of the Avp enhancer, which then in turn contribute to the heightened Avp gene expression and altered HPA axis stress response [69].

Other genes that are also affected by early stress include the CRF gene, the pro-opiomelanocortin (POMC) gene coding for ACTH and the HSD11B2 gene. A chronic variable daily stress regimen in mice was found to be associated with hypomethylation of specific CpGs within the regulatory region of the CRF gene in the hypothalamus and amygdala as well as depressive-like behaviours [126]. The POMC gene that encodes CRH is a downstream target of Avp and CRH signalling. Following prenatal stress (for instance, anoxia), a number of studies have found hypermethylation of the POMC promoter region, which leads to gene expression silencing (reviewed in [82]). Finally, Harris & Seckl [3] have shown that methylation of CpG islands within the promoter and exon 1 region of the HSD11B2 gene is associated with differential expression levels of the HSD2 enzyme, thereby impacting on the amount of GCs reaching the foetus during pregnancy.

In conclusion, there is ample evidence implicating DNA methylation of specific HPA axis-related genes in the programming of the altered stress response we so often observe following repeated early stress in rodents, non-human primates and humans.

(i) Interplay of 5-HT system hypothalamic–pituitary–adrenal axis

A schematic model displaying the changes in DNA methylation of SLC6A4, GR and Avp in depression, and resulting effects on 5-HT HPA activity, is displayed in figure 1. The SLC6A4, NR3C1 and Avp genes appear most important, but further research is necessary to understand the interplay of these genes and others involved, the role of mediators and moderators and their effects on different brain regions.

5. Discussion

(a) What is the contributing role of DNA methylation processes in existing neurobiological theories of depression?

Initial evidence using the maternal care rat model elucidated a pathway for how early life factors could affect the long-term programming of gene expression. More recent animal studies, including those in non-human primates, confirmed and extended these observations, though the number of studies investigating DNA methylation processes is far fewer than studies on sequence variations and polymorphisms, and many of these methylation studies are focusing on the impact of the environment on gene expression. In regards to 5-HT, in investigating different SLC6A4 promoter regions and using different methods for methylation analysis, they have demonstrated an association between peripheral SLC6A4 methylation and various depression-relevant behaviours in which the central 5-HT system has been implicated.

In regards to the HPA axis, studies have consistently shown that maternal care has long-lasting effects on the expression of both the gene encoding for the GC receptor, NR3C1, and the Avp enhancer, with associated anxious- and/or depressive-like behaviours in rodents, non-human primates and humans. Presumably, early repeated stress can impact one or both of these genes, as well as other HPA axis-relevant genes such as the POMC, and this altered gene expression renders the organism vulnerable to subsequent stressors.

Although DNA methylation is potentially reversible by enzymatic processes, research in animals has shown that the early environment programmes DNA methylation and other epigenetic marks, and that this programme remains stable later in life [127]. Hence, these observations indicate that DNA methylation is likely a molecular mechanism through which genes and the early environment interact.

Interestingly, it appears that the effects of the environment on DNA methylation may be partly dependent on gene sequence. A recent review suggested that genotypes or alleles could differ in their sensitivity to the environment. With regards to SLC6A4 for instance, it could be argued that the s allele of the SLC6A4 gene is not necessarily directly linked to a disorder (e.g. depression), but is more sensitive to the negative consequences of adversity as well as to the effects of a positive stimulating or enriching environment and/or social support [138]. Hence, if the s allele and/or ss genotype is more vulnerable to environmental insults, the methylation levels could differ by polymorphism or
Table 2. Human epigenetic studies of HPA axis-related genes.

<table>
<thead>
<tr>
<th>topic</th>
<th>reference</th>
<th>species and cells</th>
<th>sample</th>
<th>method</th>
<th>findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>suicide with history of childhood abuse</td>
<td>[136]</td>
<td>rRNA promoter from human brain tissue</td>
<td>18 suicide victims and 12 controls, all males</td>
<td>sodium bisulphite mapping</td>
<td>hypermethylation of rRNA promoter in the hippocampus but not cerebellum in suicide victims with childhood abuse</td>
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<tr>
<td></td>
<td></td>
<td>human hippocampal tissue</td>
<td>12 suicide victims with positive history of childhood abuse, 12 suicide victims with negative history and 12 controls</td>
<td>sodium bisulphite mapping</td>
<td>enhanced DNA methylation in the NR3C1 exon 1b promoter and decreased NR3C1 mRNA expression in suicide victims with a history of childhood abuse, compared with suicide victims without childhood abuse and controls</td>
</tr>
<tr>
<td></td>
<td>[135]</td>
<td>human hippocampal and anterior cingulate</td>
<td>21 suicide victims with positive history of childhood abuse, 21 suicide victims with negative history and 14 controls</td>
<td>quantitative reverse-transcriptase polymerase chain reaction; pyrosequencing</td>
<td>hippocampal expression of NR3C1 variants 1b, 1c and 1d was decreased in suicide victims with a history of childhood abuse, compared with suicide victims without childhood abuse and controls. No group differences in the anterior cingulate</td>
</tr>
<tr>
<td>major depression</td>
<td>[130]</td>
<td>human cord blood monocytes</td>
<td>13 infants with depressed mother (untreated), 33 infants with depressed mother treated with 5-HT reuptake inhibitor (n = 33) and 36 infants with non-depressed mothers</td>
<td>pyrosequencing</td>
<td>increased methylation of NR3C1 at a predicted NGFI-A binding site was associated with prenatal exposure to maternal depressed mood in the third semester. Increased methylation was also associated with increased salivary cortisol stress responses at three months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>human brain tissue</td>
<td>6 depressed patients and 6 controls</td>
<td>PCR and sodium bisulphate mapping</td>
<td>decreased GRα in amygdala, cingulate gyrus and inferior PFC; decreased NGFI-A in hippocampus in depressed patients compared with controls</td>
</tr>
</tbody>
</table>


genotype. Indeed, in monkeys, greater methylation levels in carriers of the s allele were found compared with carriers of the l allele. Notably, in humans, one study found that the direction of the association between the level of SLC6A4 methylation and early life stress depends on genotype, with positive associations in the Il carriers and negative associations in ss carriers [124]. It could thus be speculated that the effects of the environment on DNA methylation could be partly dependent on the genotype, which would then affect the brain and behaviour differently. Future studies should further investigate the impact of adversity on DNA methylation patterns as a function of genotype.

Although it is generally accepted that stressors increase vulnerability for the development of depression, it is important to mention that an adverse outcome is not written in stone. It is clear that the actual outcome depends on the interaction with other environmental factors as well as genetic factors involved in stress adaptation. Many studies have supported the traditional ‘cumulative stress’ hypothesis, postulating that acute or chronic stressful experiences in later life will add to the already programmed early life vulnerability and thereby increase disease risk [139,140]. There is however, also accumulating evidence for the so-called ‘mismatch’ hypothesis, implying that the early environment predicts problems only when there is a high degree of mismatch either between the early life and the actual adult environment [139–142], or between the pre and postnatal environment [75]. Hence, an individual who has experienced high levels of early or prenatal adversity is adapted to deal with high levels of stress in adulthood or postnatally and is therefore ‘better off’ with exposure to high stress levels in later life [139–142]. The role of epigenetic factors in the behavioural translation of such mismatch is presently unknown. Although there is an ongoing debate about individual factors that favour the first or second hypothesis [139,140], both views strongly support the need for longitudinal study designs when extrapolating the findings of experimental epigenetic studies in animals to human samples, since such a design is the only way to investigate interactions between various stressors throughout the lifespan reliably.

(ii) Is the epigenetic response to low maternal care specific to particular genes?

The observation of altered DNA methylation based on maternal care was made using the candidate gene approach. However, the large number of phenotypes that are associated with early life adversity in both animals and humans suggest that maternal care can have a broad effect in different brain regions involving numerous mediators. Indeed, by performing microarray analyses in the hippocampus of adult rats, it was found that maternal care has a broad impact on gene expression and DNA methylation [145]. Interestingly, the DNA methylation response to maternal care was found in clusters of not only gene promoters but also bodies of genes and intragenic sequences, as well as those residing distantly from transcriptional start sites and regions containing non-coding RNAs [145]. In other regions of the brain, candidate genes such as brain-derived neurotrophic factor (BDNF) were found to be epigenetically modulated in the PFC of rats by maternal behaviour and the effect is lifelong and trans-generational [146].

(iii) What does methylation in peripheral tissues tell us about the brain?

An important methodological concern in assessing methylation in relation to mental health problems including depression is that it is impossible to measure DNA methylation directly in the living human brain.

DNA methylation patterns of some genes are highly tissue-specific, thus it is clear that the methylation pattern of a gene in a peripheral tissue such as white blood cells may not necessarily reflect the state of methylation of the same gene in the brain. This concern should however not dissuade researchers from epigenetic analysis of behavioural problems in living humans since recent studies have suggested that methylation patterns are informative in a behavioural context. In addition, although some genes show tissue-specific DNA methylation patterns, others are similarly methylated in peripheral cells and the brain. We propose that the link between the DNA methylation patterns in the immune system and behaviour is threefold. First, T cells and other lymphocytes communicate with the central nervous system (CNS) through cytokines such as IL-1β. The cytokine IL-1β is expressed in peripheral blood cells, but may be due to the use of different early stress paradigms.

Figure 1. The HPA axis and the 5-HT system and associated DNA methylation changes in depression.
receptors for IL-1 β are present in the brain. Given the link between HPA axis activity and IL-1β expression, differences in IL-1β are likely to account for differences in stress-responsiveness as well as related behaviours [147]. Second, although the gene candidates are different in the brain and peripheral cells, the response to early life adversity can be observed in both central and peripheral systems. Thus, although the methylation response in peripheral cells is not expected to be identical to the response in different regions of the brain, the methylation readout would support the hypothesis that adversity elicits an epigenetic response and would identify individuals who had developed a systemic response to adversity. Third, certain genes expressed in both peripheral cells and the brain could respond to adversity in a similar direction. Thus, for certain genes the methylation pattern in peripheral cells might predict similar changes in the brain.

Further support for the relevance of peripheral methylation for brain function comes from studies showing that certain genes are similarly altered in both peripheral tissue and the brain. BDNF, a key neurotrophic factor in the brain that is linked with stress response and depression, is an example [148]. A study in mice hippocampi has found decreased BDNF mRNA in association with depression-like behaviours and DNA hypermethylation of the BDNF promoter IV [149]. In humans, the methylation status of the BDNF promoter (exon 1) in peripheral blood has been shown to differentiate healthy individuals and patients with major depression [150]. Membrane-bound catechol-O-methyltransferase (MB-COMT) is another example. In post-mortem human brains, hypomethylation of the MB-COMT promoter and increased gene expression were associated with both schizophrenia and bipolar disorder [151]. In the periphery, hypomethylation of MB-COMT was observed in DNA obtained from saliva in psychiatric patients, suggesting that peripheral DNA methylation may potentially serve as a screening tool for schizophrenia and bipolar disorders [152]. Furthermore, studies that examined DNA methylation of genes in multiple human tissues revealed that peripheral blood-derived DNA shares a similar methylation pattern as DNA found in the brain for certain genes [153,154].

With regards to SLC6A4, immune cells are desirable because they can be easily obtained for diagnosis [155]. Studies have found positive correlations for SLC6A4 kinetics between blood platelet and nerve endings, midbrain and brain synaptosomes, suggesting an association between peripheral and central SLC6A4 activity. [156–158]. Moreover, it is possible to use peripheral blood to detect psychiatric conditions involving central 5-HT alterations. Numerous studies have used SLC6A4 alterations in blood lymphocytes and platelets in a variety of mental disorders, including schizophrenia, bipolar disorder and anxiety disorders. [159–161]. Finally, SLC6A4 methylation studies were able to describe associations between methylation of blood-derived DNA and depression and alcoholism in human [121,122].

It is also important to mention that SLC6A4 gene expression can be sensitive to various physiological influences, such as HPA activity. For instance, glucocorticoids can cause an increase in SLC6A4 expression in human B-lymphoblastoid cells [162]. Furthermore, quantification of the readily degrading mRNA depends greatly on the sample processing time [163]. In contrast, methylation remains more constant over time. This makes methylation of SLC6A4 a more reliable biomarker than, for example, RNA [163].

The relevance of DNA methylation processes is further demonstrated by a recent pilot study in which we investigated the correlation between DNA methylation states (in monocytes and T cells) of the SLC6A4 promoter at age 27 years and in vivo measures (positron emission tomography, PET) of brain 5-HT synthesis. We measured DNA methylation 360–710 bp upstream of the SLC6A4 promoter [164] and found an association between in vivo measures of 5-HT and DNA methylation. We thus demonstrated, for the first time, an association between peripheral white blood cell DNA methylation and 5-HT synthesis in the brain. Studies are currently ongoing to replicate and expand upon these findings.

(c) Suggestions for future research
Though DNA methylation is potentially reversible by enzymatic processes, it is the most stable epigenetic mark as it is part of the chemical structure of the DNA itself, and thus has excellent diagnostic potential as a biomarker of altered genomic functions [147,165]. This diagnostic potential of DNA methylation is well developed in oncology but has not been applied as of yet to behavioural states. In addition, epigenetic studies in relation to mental health problems, including depression, are so far correlational and thus do not provide any insight into cause and effect. Longitudinal studies in patients and at-risk samples are needed to study the diagnostic predictive value of DNA methylation reliably.

DNA methylation profiles may also eventually be used to assess treatment response. For instance, there is some evidence that methylation alterations may underlie the therapeutic mechanism of antidepressants. For instance, a recent study found higher DNA methylation in the promoter region of P11 in a rodent model of depression and demonstrated that this hypermethylation profile could be reversed by chronic use of the selective serotonin uptake inhibitor (SSRI) citalopram [166]. Given the role of SLC6A4 in the therapeutic efficacy of SSRIs, it would be of interest to investigate whether the state of DNA methylation in this gene could serve as a marker for assessing the efficacy of antidepressant therapy upon follow-up. Investigation of DNA methylation processes before and after an intervention could also shed light on mechanisms/causality.

A crucial issue in attempting to understand environmental effects on epigenetic programming is to differentiate genetic from environmental effects. Notwithstanding environmentally induced alterations, gene expression is heritable and under genetic control. Hence, it is important to determine to what extent epigenetic differences are reflections of the genotype (i.e. differential impact of exposure to environmental risk factors according to genotype or a G × E correlation) or a true marker of environmental exposures. Monozygotic (MZ) twins are ideal subjects to study environmental effects because they share the same genes. The few studies that investigated epigenetic differences within MZ twin pairs showed remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation, affecting their gene expression profile [167,168]. In MZ twins, correlations in epigenetic markers are moderate, ranging from 0.30 to 0.42 [169]. These correlations suggest that gene expression is in large part determined by environmental factors, but that it is important to also take the influence of DNA sequence polymorphisms into account. Interestingly, the impact of environmental conditions on DNA methylation can also be studied in individuals that
have gone through changes in early family environments as part of mental health preventive interventions [170]. This type of observational study may be viewed as the human equivalent of the cross-fostering experiments with rats [127] and non-human primates [171].

6. Conclusion

To conclude, present research studying G × E interactions in relation to depression supports the need to go beyond the traditional G × E models that have simply taken into account genetic polymorphisms (i.e. fixed DNA sequence). DNA methylation may be an important underlying physiological mechanism of how G × E interactions can translate into depression. However, future large-scale studies with longitudinal samples, and studies investigating the effects of experimental manipulations of the environment on epigenetic processes are needed [172]. More methodologically rigorous studies should help to explain some of the inconsistencies in the epigenetic research conducted in humans. In the long run, epigenetic research may favour the early identification of individuals at risk for depression as well as help identifying effective preventive and corrective interventions.

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