

# Glucocorticoids and fetal programming part 2: mechanisms

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**Abstract** | The lifelong health of an individual is shaped during critical periods of development. The fetus is particularly susceptible to internal and external stimuli, many of which can alter developmental trajectories and subsequent susceptibility to disease. Glucocorticoids are critical in normal development of the fetus, as they are involved in the growth and maturation of many organ systems. The surge in fetal glucocorticoid levels that occurs in most mammalian species over the last few days of pregnancy is an important developmental switch leading to fundamental changes in gene regulation in many organs, including the brain. These changes are important for the transition to postnatal life. Exposure of the fetus to increased levels of glucocorticoids, resulting from maternal stress or treatment with synthetic glucocorticoids, can lead to long-term ‘programming’ of hypothalamic–pituitary–adrenal function and behaviours. Glucocorticoids act at multiple levels within the fetal brain. Growing evidence indicates that they can exert powerful effects on the epigenome, including on DNA methylation, histone acetylation and microRNA, to influence gene expression. Such influences probably represent a critical component of the ‘programming’ process, and might be partly responsible for the transgenerational effects of antenatal glucocorticoid exposure on neurologic, cardiovascular and metabolic function.

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## Introduction

The early environment shapes physiological function throughout life. In rats, experience in the neonatal period can affect hypothalamic–pituitary–adrenal (HPA) responsiveness to stress in adulthood.<sup>1</sup> Findings in a range of species suggest that neurologic, endocrine, metabolic and cardiovascular function and dysfunction in adulthood have developmental origins. In addition, many studies have linked altered placental morphology, low birthweight and accelerated growth in early childhood with the development of a range of adult pathologies.<sup>2</sup> These pathologies begin to emerge in childhood, and some individuals are more vulnerable than others; thus, early interventions to prevent the development of disease might be possible. This field of research is referred to as the ‘developmental origins of health and disease’. Technological advances have enabled the investigation of the core mechanisms involved in developmental ‘programming’ of adult health and disease.

The first part of this Review discussed evidence from human and animal studies showing that synthetic and endogenous glucocorticoids affect fetal programming.<sup>3</sup> In this part of the Review, we focus on the mechanisms underlying fetal glucocorticoid programming. We discuss epigenetic modifications, the effects of maternal stress and synthetic glucocorticoids (sGC) and mechanisms of transgenerational programming.

## Glucocorticoids in the mother and fetus Transfer from mother to fetus

In considering the mechanisms by which sGC and prenatal stress program the developing fetus, it is first important to consider the signals that are passed from mother to fetus (and particularly to the fetal brain) under these circumstances (Figure 1). Previous studies have shown that sGC readily cross the placenta and enter the fetal brain, where they downregulate central drive (through glucocorticoid feedback mechanisms) to the fetal HPA axis and have a direct effect on brain development (for example neurogenesis, synaptogenesis and myelination).<sup>4</sup> By contrast, endogenous glucocorticoids (such as cortisol and corticosterone) do not pass as readily from the maternal to fetal circulation, due to the presence of placental 11 $\beta$ -hydroxysteroid dehydrogenase (HSD).<sup>5</sup> This enzyme has two distinct isoforms; 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2. 11 $\beta$ -HSD1 has both dehydrogenase (cortisol to cortisone) and reductase (cortisone to cortisol) activities, and is widely expressed in mammalian tissues, most notably the liver. By contrast, 11 $\beta$ -HSD2 only has dehydrogenase activity, has a higher affinity for glucocorticoids than 11 $\beta$ -HSD1, and its expression is highly tissue-specific, including the placenta and kidney. In the placenta, 11 $\beta$ -HSD2 converts maternal cortisol to inactive cortisone, which creates an effective gradient between mother and fetus (Figure 2). However, the barrier is not complete, and studies in guinea pigs have clearly demonstrated that maternal stress results in an incremental increase in levels of cortisol in the fetal circulation.<sup>6</sup> Of note, a reduction in

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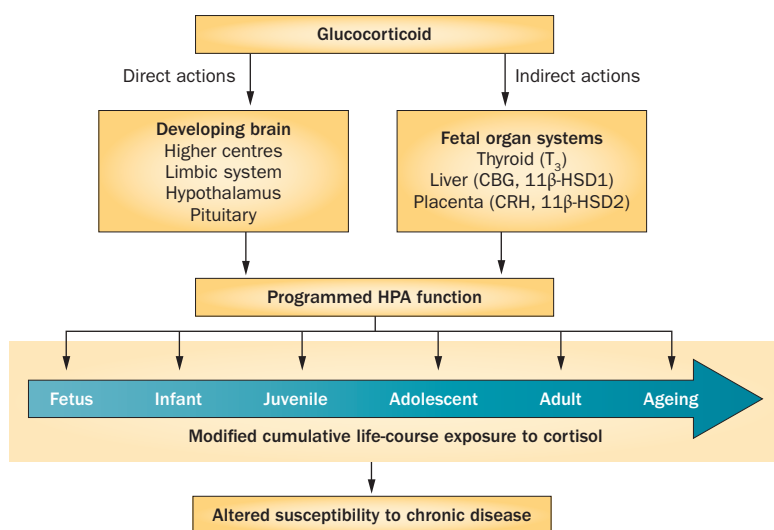
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## Competing interests

The authors declare no competing interests.

### Key points

- Transporter proteins and enzymes in the placenta and fetus protect the fetus from exposure to high levels of glucocorticoid; however, expression of these factors changes as a function of gestational age
- Differences exist in the mechanisms by which synthetic glucocorticoids and endogenous glucocorticoids (that is, cortisol) affect fetal brain development, due to differences in receptor activation
- Prenatal stress and glucocorticoids can influence the developing epigenome in a number of ways, including modified DNA methylation, histone acetylation and microRNA expression
- Glucocorticoids probably act via a number of direct and indirect routes to influence the developing epigenome
- Programmed changes in hypothalamic–pituitary–adrenal function and other endocrine systems following antenatal glucocorticoid exposure probably continue interacting with the epigenome throughout life
- The latest evidence indicates that transgenerational epigenetic transmission might be partly responsible for the multigenerational effects of synthetic glucocorticoid exposure and maternal stress in pregnancy



**Figure 1** | The routes by which glucocorticoids can program the HPA axis. Glucocorticoids might have direct or indirect effects on the developing brain. Glucocorticoids are known to modify production of  $T_3$  from the thyroid, alter expression of CBG and 11 $\beta$ -HSD1 in the liver, and modify CRH and 11 $\beta$ -HSD2 levels in the placenta. These factors will alter the levels of thyroid hormones and cortisol in the fetus and might indirectly affect the developing HPA axis. Long-term changes in HPA function will modify cumulative body exposure to cortisol throughout life, which is known to alter susceptibility to chronic disease. Abbreviations: 11 $\beta$ -HSD, 11 $\beta$ -hydroxysteroid dehydrogenase; CBG, corticosteroid binding globulin; CRH, corticotropin-releasing hormone; HPA, hypothalamic–pituitary–adrenal.

placental levels of 11 $\beta$ -HSD2 occurs during late gestation in many species, which enables increased transfer of active glucocorticoid from mother to fetus.<sup>7</sup>

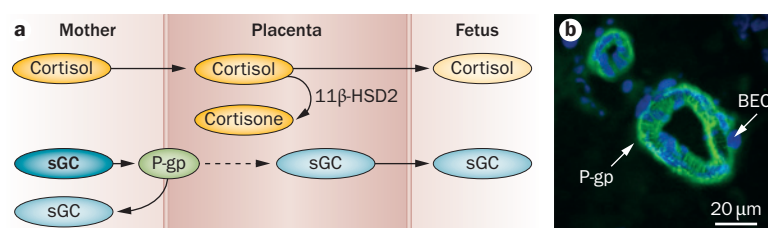
Placental 11 $\beta$ -HSD2 has a low affinity for sGC, and thus does not provide an effective barrier between mother and fetus. However, the latest evidence indicates that the placenta might be involved in decreasing the transfer of sGC from mother to fetus.<sup>8,9</sup> P-glycoprotein, a member of the ABC transporter family, is expressed at high levels in the syncytiotrophoblast layer of the mammalian placenta.<sup>8,9</sup> This transporter has a wide range of substrate specificity for xenobiotic products, drugs and certain hormones; P-glycoprotein is a particularly effective transporter of sGC.<sup>10</sup> As such, P-glycoprotein probably has

an important protective role in reducing the transfer of sGC from the maternal to fetal circulations. Importantly, P-glycoprotein is also expressed at high levels in the fetal blood–brain barrier, where it provides an additional level of protection for the fetal brain (Figure 2).<sup>11</sup> In late gestation, a marked decrease in placental levels of P-glycoprotein occurs and a corresponding increase in levels of P-glycoprotein in endothelial cells of the developing blood–brain barrier is observed.<sup>8,11</sup> Changes in the placental levels of 11 $\beta$ -HSD2 and P-glycoprotein during the course of gestation will have a considerable effect on the amounts of endogenous glucocorticoid and sGC that are transferred from mother to fetus, which could partly account for variable effects of maternal stress and treatment with sGC on fetal programming when administered at different stages of gestation.

In addition to placental modulation of glucocorticoid transfer from the mother to the fetus, glucocorticoids can exhibit direct actions on placental function, which indirectly influence fetal development. In humans, both sGC and endogenous glucocorticoids can increase production and release of corticotropin-releasing hormone (CRH) in the placenta, which in turn can activate the fetal and maternal HPA axes.<sup>12</sup> Importantly, nonprimate species do not generally express CRH in the placenta, which might account for some of the species differences identified following increased prenatal exposure to glucocorticoids (endogenous or exogenous). Glucocorticoids can also result in placental growth restriction; however, the size of the effect depends on the species, the amount and/or dose and the point in gestation at which exposure or administration occurs. In addition, exposure to sGC and endogenous glucocorticoids can inhibit production of placental lactogen and alter placental vascularization and structure, as well as placental nutrient transfer.<sup>13</sup> These changes will affect normal fetal development and probably represent another indirect route by which glucocorticoids can ‘program’ HPA function. Another important consideration is that of sex differences in the placenta. Elegant studies have identified considerable sex differences in function and responsiveness of the placenta to challenge (for example, pre-eclampsia and preterm labour).<sup>14</sup> Such differences might account for the considerable sexual dimorphism that has been associated with fetal programming after prenatal glucocorticoid exposure.<sup>14</sup>

### Effects in the brain

Once in the brain, glucocorticoids affect many aspects of neurogenesis and gliogenesis. These effects are greatest in brain structures that contain the highest levels of glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), such as the limbic system, hypothalamus and cortex; these are the same areas that are critical for the regulation of HPA function.<sup>15</sup> Glucocorticoids also affect axonal and dendritic development and synaptogenesis.<sup>16,17</sup> Importantly, both GR and MR are expressed at high levels in the fetal brain of many species, including humans.<sup>18</sup> Their expression is highly region-specific and changes dynamically with advancing gestation.



**Figure 2** | Enzymatic and transport barriers in the placenta and fetal brain.

**a** | High levels of maternal cortisol are prevented from entering fetal circulation through inactivation to cortisone by  $11\beta$ -HSD2 in trophoblast cells. sGC in maternal circulation are removed from trophoblast cells by the efflux transporter P-gp.

**b** | Fluorescent immunostaining of P-gp protein in brain microvessels derived from a fetal guinea pig. P-gp protects the developing brain from exposure to excess endogenous and synthetic glucocorticoids by pumping them from BECs back into the circulation. Nuclei from BECs are stained blue with DAPI, and P-gp protein is stained green. Abbreviations: BECs, brain endothelial cells; P-gp, P-glycoprotein; sGC, synthetic glucocorticoids.

These region-specific changes in the expression of the corticosteroid receptors lead to altered local sensitivity to both sGC and endogenous glucocorticoids.<sup>19</sup> Another important point to consider is that of receptor selectivity. The sGC (such as betamethasone and dexamethasone) bind to GR but not MR. By contrast, endogenous glucocorticoids (for example, cortisol and corticosterone) bind to both GR and MR.<sup>20</sup> This selectivity represents a fundamental difference between the programming effects of sGC and those of prenatal stress associated with raised levels of endogenous glucocorticoids. In the case of sGC, GR in the brain and pituitary are occupied, which leads to feedback inhibition of the maternal and fetal HPA axes and a reduction in levels of cortisol (or corticosterone). Under these circumstances, MR in the developing hippocampus become 'starved' of ligand and respond by upregulating the expression of MR.<sup>4</sup> This situation is quite different from that in prenatal stress, in which fetal plasma levels of cortisol are raised and cortisol binds both GR and MR. In addition, sGC interact with other orphan nuclear receptors in the brain, such as the nuclear receptor subfamily 1 group I member 2 (previously known as pregnane X receptor), and in doing so might indirectly affect brain development.<sup>21</sup> These important differences between the actions of sGC and endogenous glucocorticoids probably account for many of the differences between programming of neuroendocrine function associated with sGC and that associated with prenatal stress.

The programming effects of sGC and maternal stress in pregnancy on the developing brain are not confined to direct or indirect actions at the placenta. Glucocorticoids are critical for maturation of several organs during fetal development, such as the thyroid and liver.<sup>22</sup> Thyroid hormone is critical for normal brain development, including maturation of neurotransmitter systems.<sup>23</sup> The liver has a critical role in the production of corticosteroid-binding globulin and produces high levels of  $11\beta$ -HSD1, both of which can have a direct effect on local and circulating concentrations of bioactive cortisol in the fetus.<sup>7</sup> As such, actions of glucocorticoids in these organs influence systems that are critically important in

development of the fetal HPA axis, and perturbations in these systems might lead to long-term programming of HPA function. Manipulation of the fetal environment also results in sex-specific alterations in the production of cortisol from extra-adrenal tissues. For example, maternal undernutrition in baboons resulted in increased levels of *HSD11B1* mRNA and cortisol in perirenal adipose from female fetuses, whereas levels of *HSD11B1* mRNA were increased in the liver of male fetuses.<sup>24</sup> The sex-specific nature of these effects might help to explain some of the differences observed between male individuals and female individuals in programming of HPA activity.

### Effects on ageing

Glucocorticoids are involved in many of the molecular processes of ageing, including cognitive impairment, muscle atrophy, immune suppression and osteoporosis.<sup>25</sup> Long-term exposure to excess levels of glucocorticoids can accelerate the ageing process, whereas long-term reductions in the levels of glucocorticoids slow the ageing process.<sup>25–27</sup> Telomeres are important for chromatin protection and telomerase is critical in the maintenance of normal telomere length.<sup>28</sup> Reduced telomerase activity has been identified in patients with chronic stress, and in cells (lymphocytes) treated with cortisol *in vitro*.<sup>28,29</sup> Glucocorticoids have been postulated to accelerate cellular ageing by reducing the length of telomeres.<sup>29</sup> Long-term changes in the level of exposure to endogenous glucocorticoids in individuals with increased HPA activity that was programmed *in utero* might, therefore, adversely affect telomere protection of chromosomes. This hypothesis is supported by the observation that embryonic exposure to excess levels of corticosterone, which resulted in a prolonged HPA response to stress in chicks, was also associated with increased levels of reactive oxygen metabolites and a reduction in overall telomere length.<sup>30</sup>

### Alterations in HPA function

As described in part 1 of this Review,<sup>3</sup> a number of routes exist by which HPA function can be altered following prenatal exposure to glucocorticoids, including modified drive to the axis and altered sensitivity of the negative feedback response to glucocorticoid exposure. Programmed increases in HPA function are associated with a reduction in negative feedback sensitivity to glucocorticoids, primarily at the level of the hippocampus.<sup>31,32</sup> However, it is possible that reduced glucocorticoid sensitivity might be programmed in other peripheral organ systems, such that while circulating levels of cortisol are increased, local glucocorticoid signalling might be reduced. In this case, the reduction in local levels of glucocorticoids might be responsible for a given pathophysiology. Conversely, where programming reduces HPA function as a result of increased glucocorticoid negative feedback sensitivity, hypersensitivity to glucocorticoids might also occur in other peripheral organ systems. These possibilities clearly require further detailed investigation.

## Mechanisms of fetal programming

### Epigenetics and glucocorticoids

In the preceding section, a number of direct and indirect routes by which glucocorticoids could program the developing brain and neuroendocrine systems have been discussed. However, it is also important to consider the specific mechanisms by which long-term programming of the function of the HPA axis and behaviour can occur. Epigenetics is receiving increased focus in the field of developmental programming. Epigenetic processes represent a route by which environmental stimuli, both external and internal to an organism, can modify gene expression. Over the past 10 years, it has become clear that epigenetic processes are involved in fetal programming of neurologic, endocrine, cardiovascular and metabolic function. For example, a number of the HPA-regulatory genes (that is, *NR3C1*, *NR3C2*, *CRH*, *POMC* and *HSD11B2*) are regulated by DNA methylation and histone acetylation.<sup>33–38</sup>

Gene expression can be modified by epigenetic processes, which in some cases can be very stable (for example, X-chromosome silencing in female individuals) or highly transient (for example, cyclical methylation of the *ESR1* [estrogen receptor] promoter).<sup>39</sup> Epigenetic modifications include DNA methylation (CpG or non-CpG), histone modifications (for example, acetylation, methylation, phosphorylation, ubiquitination and sumoylation) as well as through short (that is, microRNA, or miRNA) or long noncoding RNAs.<sup>40</sup> These modifications can occur anywhere in the genome; however, they often occur in enhancers, promoters, exons and imprinted control regions. In the context of developmental programming, the majority of studies have focused on changes in DNA methylation and chromatin structure (for example, histone acetylation). However, findings published in 2013 have also identified the importance of miRNA in transgenerational programming.<sup>41</sup> DNA methylation in regulatory regions of genes such as promoters and enhancers is most often associated with a reduction in gene expression. By contrast, gene activation often results from demethylation of the promoter.<sup>42</sup> After methylation of a promoter, the methyl group can prevent binding of transcription factors and subsequent transcription. Promoter methylation can also lead to gene silencing through association of DNA binding proteins, such as MeCP-1 and MeCP-2 (methyl cytosine binding proteins 1 and 2). Once bound to the methylated promoter, these factors recruit co-repressors and histone modifying enzymes, including histone deacetylases and histone methyltransferases, resulting in an inactive chromatin configuration and a reduction in transcription.<sup>42</sup>

Studies in humans and in animal models have identified that maternal treatment with sGC,<sup>43</sup> prenatal stress,<sup>36,37</sup> dietary manipulation during pregnancy<sup>44,45</sup> and altered levels of maternal care<sup>33,46,47</sup> leave epigenetic marks on the DNA of offspring. These marks have been linked with long-term changes in gene expression. An elegant series of studies in rats have shown that increased levels of maternal care lead to demethylation

and increased histone acetylation of the hippocampal *Nr3c1* promoter, an effect that is maintained throughout life.<sup>33</sup> These epigenetic modifications lead to increased hippocampal expression of *Nr3c1* mRNA and GR.<sup>33</sup> In this model, increased levels of GR were associated with an increase in glucocorticoid-induced negative feedback, resulting in decreased HPA activity.<sup>47</sup> The route by which alterations in methylation of the *Nr3c1* promoter occur in this model has also been determined. Increased levels of maternal care are associated with increases in hippocampal levels of serotonin (that are dependent on levels of thyroid hormone), which result in increased *Egr1* transcription.<sup>48</sup> This initial increase in levels of NGFI-A protein has been postulated to actively recruit a histone acetylase transferase (CREB-binding protein), increasing acetylation and accessibility to the DNA demethylase MBD2, which results in stable upregulation of *Nr3c1* gene expression.<sup>47,48</sup> The same group have also shown increased hippocampal *NR3C1* promoter methylation in people who had committed suicide and had also experienced childhood abuse.<sup>46,49</sup>

### Maternal stress and epigenetic modification

Maternal stress in pregnancy leads to acute and long-term epigenetic modification of the genes that encode proteins involved in regulating the HPA axis. In mice, stress in early pregnancy (which led to increased HPA responsiveness to stress in male offspring) was associated with reduced DNA methylation of the hypothalamic *Crh* promoter and increased methylation of the hippocampal *Nr3c1* promoter. The latter corresponded with a decrease in hippocampal expression of *Nr3c1*.<sup>36</sup> Maternal stress in late gestation also resulted in altered expression of a number of miRNAs in the brains of newborn rats.<sup>50</sup> This study utilized the whole brain for analysis, which restricts the functional interpretation. However, a study published in 2013 showed that prenatal stress altered the expression of a number of miRNAs in the hippocampus and prefrontal cortex of juvenile and adult rat offspring.<sup>51</sup> Another important study in rats has demonstrated that maternal stress in late gestation increased DNA methylation in the placental *Hsd11b2* promoter, which was associated with increased placental expression of the DNA methyltransferase *Dnmt3a*. The increased methylation of the *Hsd11b2* promoter in the placenta corresponded to a decrease in levels of *Hsd11b2* mRNA.<sup>52</sup> This decrease would probably result in downregulation of levels of the 11 $\beta$ -HSD2 enzyme in the placenta and increased transfer of maternal endogenous glucocorticoid to the fetus, exacerbating the effects of prenatal stress on the fetus.

In humans, maternal depression and/or anxiety in the third trimester is associated with increased methylation of the *NR3C1* promoter (at exon 1F) at an NGFI-A binding site in genomic DNA from umbilical mononuclear cells.<sup>37</sup> This increased methylation was associated with an increased cortisol response to a stressor in infants at 3 months of age.<sup>37</sup> A study undertaken in women experiencing stress under war conditions demonstrated a strong correlation between maternal stress



in pregnancy and increased *NR3C1* promoter methylation in genomic DNA derived from umbilical cord blood at birth.<sup>53</sup> Together, these data suggest that similar epigenetic modification to those identified in animal models may occur in humans.

### sGC and epigenetic modification

Few studies have investigated the effects of sGC on the developing epigenome. An early study demonstrated that long-term *in vitro* treatment of rat hepatoma cells with dexamethasone resulted in stable DNA demethylation within a key enhancer region of the gene that encodes the liver-specific tyrosine aminotransferase in rats (*Tat*).<sup>54</sup> The same region of the *Tat* enhancer is methylated in the fetal rat liver, and demethylation occurs co-incident with the late gestation surge in levels of free corticosterone in the fetal circulation. Furthermore, this demethylation could be induced prematurely by exposure of fetal liver cells to dexamethasone.<sup>54</sup> This association between the late gestation glucocorticoid surge and changes in the developing epigenome has been investigated in multiple fetal organ systems, including the whole brain and hippocampus of guinea pigs.<sup>43,55,56</sup> At the level of global DNA methylation, the surge of cortisol levels in late gestation was associated with a decrease in methylation in the adrenal glands and placenta, but an increase in methylation in the kidney.<sup>43</sup> These changes in global DNA methylation were associated with substantial changes in the expression of a number of key enzymes involved in regulation of the epigenetic state (including DNA methyltransferases and methyl CpG-binding domain proteins). In the hippocampus, the surge in levels of cortisol during late gestation was associated with dramatic changes in genome-wide transcription and promoter methylation.<sup>55,56</sup> These studies provide strong evidence that the natural glucocorticoid surge in late gestation is an important epigenetic 'switch' in fetal organs, including the brain, which leads to profound developmental changes in gene expression.

Studies published in the past 2 years have focused on the effects of maternal treatment with sGC in late gestation.<sup>43,55,56</sup> In the guinea pig, the profile of global DNA methylation in the fetal adrenal, kidney and placenta after multiple-course antenatal sGC exposure (gestational day [GD] 50, before the natural cortisol surge) are the same as those associated with the natural cortisol surge that occurs between GD 50 and GD 65.<sup>43</sup> This finding further implicates glucocorticoids as an important epigenetic 'switch' in the fetus during late gestation. The effects of sGC on the developing epigenome remain after birth, with altered global levels of DNA demethylation in the brain, adrenal and liver of adult offspring.<sup>43</sup> This observation clearly demonstrates that fetal exposure to sGC has lifelong effects on the epigenome; however, the specific promoters affected remain to be determined.

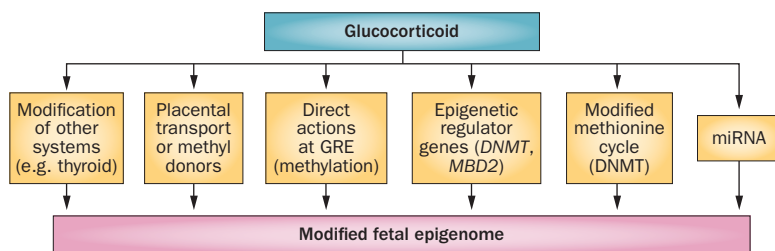
In the fetal hippocampus, maternal exposure to sGC resulted in dramatic changes in gene transcription, promoter methylation and acetylation.<sup>55,56</sup> At the level of promoter DNA methylation, exposure to sGC was

predominantly associated with demethylation. Importantly, the genes affected by early exposure to sGC (before the natural cortisol surge) were quite different from those altered in association with the natural cortisol surge.<sup>55,56</sup> This finding suggests that, firstly, the effects of sGC on the epigenome are different to those of endogenous glucocorticoids, and secondly, that the effects of glucocorticoids on the hippocampal epigenome are highly dependent on the stage of development at exposure.

### Mechanisms of epigenetic programming

A number of routes exist by which the early environment can program the developing epigenome, leading to life-long changes in gene regulation.<sup>57</sup> However, the first and most important question pertains to whether the effects of exposure to glucocorticoids (either endogenous or exogenous) are permanent, transient or both. The early environment might leave permanent epigenetic marks that alter gene function and the resulting phenotype throughout life. Whilst a number of studies have shown that it is possible to prevent or reverse (in later life) these epigenetic marks through nutritional (such as folic acid) and pharmacological (for example, L-methionine) intervention, it is generally accepted that without intervention, the early epigenetic effects will remain.<sup>58,59</sup> However, studies have now begun to demonstrate that although sGC certainly have long-term effects on the epigenome, these changes are modified over time. For example, prenatal exposure to sGC in the fetal guinea pig resulted in an acute and substantial effect on DNA methylation and H3K9 acetylation in the hippocampus. However, after 14 days, the epigenetic landscape of the fetal hippocampus was substantially different to that identified 24 h after treatment.<sup>55</sup> As such, the initial stimulus seems to cause a wave of epigenetic modification that continues to change with time. This observation highlights the importance of determining the longitudinal profile of epigenetic changes when attempting to determine the specific mechanisms involved.

Glucocorticoids modify the epigenome through a number of routes (Figure 3). The most direct route is that of glucocorticoids binding to a glucocorticoid response element (GRE) in a gene promoter and altering local methylation. In this regard, strong evidence suggests that binding of the steroid hormone receptor to its recognition element (that is, GRE) targets demethylation.<sup>60</sup> Use of GR-chromatin immunoprecipitation has demonstrated that demethylation does occur in the promoters of a number of fetal hippocampal genes that contain GREs, following prenatal exposure to sGC in guinea pigs.<sup>56</sup> Many potential indirect routes also exist by which glucocorticoids might modify the developing epigenome. Studies in guinea pigs have shown that sGC treatment leads to acute and long-term changes in the expression of 'epigenetic regulator' genes, including *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Mbd2* and *Mecp2*, as well as a number of miRNA in several tissues, including the brain and hippocampus.<sup>43,56</sup> Another potential indirect route by which sGC treatment might lead to altered DNA methylation in the fetus is through effects on



**Figure 3** | Potential routes by which glucocorticoids influence the fetal epigenome. Glucocorticoids modify the development of other organ systems (for example, the thyroid gland), which might in turn influence the fetal epigenome. Glucocorticoids can modify the expression of placental transporters and alter the transfer of methyl donors to the fetus. There are direct actions of glucocorticoids at the GRE that result in changes in DNA methylation. Glucocorticoids can cause long-term changes in the expression of 'epigenetic regulator genes' (for example, *DNMT* and *MBD2*). Exposure to glucocorticoids can lead to hyperhomocysteinaemia which, in turn, can lead to altered DNMT activity. miRNA can also be influenced by glucocorticoids and maternal stress. Abbreviations: DNMT, DNA methyltransferases; GRE, glucocorticoid response element; MBD2, methyl CpG-binding domain protein 2; miRNA, microRNA.

the methionine cycle. In this regard, hypercortisolism associated with Cushing syndrome leads to hyperhomocysteinaemia,<sup>61</sup> which in turn results in increased levels of S-adenosylhomocysteine and a reduction in the S-adenosylmethionine to S-adenosylhomocysteine ratio. Such a reduction in this ratio can inhibit DNA methyltransferase activity, which leads to promoter demethylation.<sup>62,63</sup> The latter route is consistent with the fact that acute sGC exposure predominantly leads to promoter demethylation.<sup>43,55</sup>

The developing epigenome might also be influenced by sGC via several indirect routes. Glucocorticoids induce a plethora of endocrine changes in the developing mother and fetus.<sup>22</sup> For example, sGC treatment leads to an increase in the fetal levels of thyroid hormone ( $T_3$ ).<sup>64</sup> This system is the same as that activated by maternal licking and grooming in the rat, and through which maturation of the ascending serotonergic system increases levels of NGFI-A and modification of *Nr3c1* promoter methylation in the neonatal hippocampus.<sup>23,47</sup> Similarly, sGC treatment during late gestation in guinea pigs led to upregulation of *EGR1* expression in the fetal cerebral cortex and hippocampus.<sup>65</sup> The availability of methyl donors such as choline, methionine and folic acid can also influence the development of the fetal epigenome.<sup>58</sup> Maternal sGC treatment during the last week of pregnancy in rats modifies placental transport of folate and choline, which leads to reduced levels of fetal methionine.<sup>66</sup> Another study has shown that increased dietary intake of choline during the third trimester of pregnancy in humans results in increased global methylation in the placenta. Importantly, choline supplementation also increased methylation of the *CRH* promoter and reduced *CRH* transcription in the placenta.<sup>67</sup> Thus, an sGC-induced change in placental and fetal methyl donor availability might profoundly influence the fetal epigenome. Clearly, glucocorticoids can have a major influence on the developing epigenome, which occurs through a number of direct and indirect pathways.

### Translation to humans

Numerous studies have demonstrated the tissue-specific nature of epigenetic modification. As such, a major limitation of translating epigenetic findings from animal studies to humans is an inability to sample the tissue of interest (such as the hippocampus, hypothalamus or pituitary). As a result, the development of potential epigenetic biomarkers in other tissues, particularly blood and saliva, has generated much interest. The latest evidence indicates that white blood cells in saliva and blood could provide biomarkers of epigenetic changes in the brain. For example, demethylation of the *COMT* and the *HTR2A* promoters occur in the brains of patients with schizophrenia.<sup>68,69</sup> Similar changes occur in *COMT* and *HTR2A* promoters in the salivary DNA of patients with schizophrenia and bipolar disorder.<sup>70,71</sup> Translation of epigenetic knowledge will aid mechanistic understanding of programming in humans, but will also potentially provide diagnostic or prognostic markers. As an example, analysis of the *NR3C1* exon 1F promoter in DNA from lymphocytes of patients with post-traumatic stress disorder revealed an association between methylation state and treatment outcome.<sup>72</sup>

### Transgenerational programming

The effects of developmental exposure to glucocorticoids (endogenous or exogenous) can be passed across generations via a number of possible routes. These include modification of maternal endocrine and cardiovascular adaptations to pregnancy, differences in maternal behaviour and care of offspring and transgenerational epigenetic transmission. A combination of these routes probably occurs. Maternal HPA activity increases throughout gestation. This increase is partly compensated for by an increase in circulating levels of corticosteroid binding globulin. Considerable progressive increases in levels of free cortisol do occur in the second and third trimesters of human pregnancy,<sup>73</sup> and similar profiles have been identified in other mammals.<sup>7</sup> Interestingly, a reduction in maternal HPA reactivity to stress occurs over the same time period.<sup>74</sup> Maternal treatment with sGC and prenatal stress modify basal and activated HPA function in adult female  $F_1$  offspring and probably alter the adaptation of the HPA axis to pregnancy. Such a modified adaptation to pregnancy would lead to altered (increased or decreased) exposure of the  $F_2$  fetus to maternal cortisol, which, in turn, could program HPA function and behaviours in the  $F_2$  offspring.

A number of cardiovascular adaptations also occur in pregnancy to promote blood supply to the placenta. Exposure to sGC and prenatal stress lead to changes in cardiovascular function in offspring and these animals (rats, sheep and vervet monkeys) probably exhibit altered cardiovascular adaptations to pregnancy and modified placental blood flow.<sup>13</sup> The change in blood flow would result in modified nutrient and oxygen delivery to the fetus, both of which lead to programming of HPA function and behaviour. Almost no studies have investigated programmed changes in pregnancy adaptation and the

associated long-term consequences. With respect to maternal behaviours, daily restraint stress over the last week of gestation results in a reduction in maternal care in rats, which modifies HPA function in adult life.<sup>75</sup> These cycles of modified adaptation to pregnancy and maternal behaviour could repeat in subsequent pregnancies, which would perpetuate transgenerational transmission through the maternal line.

Evidence is rapidly growing to suggest that epigenetic transmission is an important mechanism in the transgenerational effects of early environmental manipulation.<sup>76–78</sup> Prenatal exposure to sGC in late gestation resulted in multigenerational (maternal transmission) reductions in global DNA methylation in the guinea pig cerebellum, liver and adrenal ( $F_1$  and  $F_2$  offspring).<sup>43</sup> In addition, sGC had multigenerational effects on the expression of ‘epigenetic regulator’ genes in these tissues (for example, *DNMT1*, *DNMT3B*, *MBD2* and *CREBBP*). Although the study in guinea pigs provides evidence of multigenerational influences of sGC on the epigenome, it does not prove that epigenetic transmission occurs. Confirmation of epigenetic transmission would require evidence of paternal transmission of the effects of antenatal sGC to  $F_3$  offspring. A number of studies have investigated transgenerational transmission of the effects of endocrine disruptors, nutrient restriction and prenatal stress through the paternal lineage.

In pregnant rats, transient exposure to the endocrine disruptor vinclozolin (a commonly used fungicide) resulted in male germline transmission of decreased spermatogenic capacity for up to four generations.<sup>79</sup> This transmission was associated with altered patterns of methylation that underwent transgenerational transmission to  $F_3$  offspring.<sup>79</sup> Vinclozolin also induces transgenerational (three generations; paternal transmission) effects on HPA function.<sup>80</sup> Other animal studies have provided additional clear evidence of epigenetic transmission or inheritance. For example, prenatal stress led to demasculinization in  $F_2$  offspring, which was transmitted via the paternal lineage.<sup>81</sup> In another study, maternal high-fat diet resulted in increased body size in  $F_3$  offspring, again via paternal transmission.<sup>82,83</sup> All of the preceding studies have described the effect of maternal manipulation in pregnancy; very few have considered the effect of paternal environmental manipulation on outcomes in offspring. Importantly, a study published in 2013 has demonstrated that offspring of fathers that were exposed to chronic stress before breeding displayed a notably reduced HPA axis responsiveness to stress and modified expression of HPA regulatory genes in the hypothalamic paraventricular nucleus.<sup>41</sup> These results certainly implicate epigenetic transmission; however, the precise mechanisms by which epigenetic transmission

might occur have remained unclear. In this regard, a transgenerational investigation using mice with a hypomorphic mutation of the enzyme methionine synthase reductase (encoded by *Mtrr*) has provided very strong evidence that epigenetic inheritance might involve methylation.<sup>78</sup> An additional study has provided evidence that paternal stress influences are mediated via altered miRNA levels in sperm.<sup>41</sup> Together, these studies would suggest that once an epigenetic defect or modification occurs, regulation of the gene might never fully revert to its original state. This situation would have clear implications for evolutionary inheritance. Further studies in this area are eagerly awaited.

## Conclusions

Maternal stress during pregnancy and fetal exposure to high levels of sGC can result in lifelong and multigenerational effects on HPA function and behaviours. Our understanding of the mechanisms of programming is rapidly expanding, with epigenetic processes clearly being implicated. However, a number of important questions remain. For example, are the epigenetic effects permanent, transient or both? Do lifelong changes in the regulation of HPA function and other endocrine systems continue to interact with the epigenome throughout life? Are there periods of development when the fetal epigenome is more sensitive or vulnerable? Are the effects of prenatal stress or fetal sGC exposure reversible, and if so, how? Can we identify meaningful epigenetic biomarkers that can be utilized in humans to identify individuals at risk? Defining the mechanisms by which prenatal stress and fetal sGC exposure lead to lifelong changes in HPA function and behaviours will perhaps enable the improvement of treatments and/or development of interventions that prevent or reverse any long-term negative consequences of fetal exposure to glucocorticoids as a result of antenatal treatment with sGC or maternal stress.

### Review criteria

Searches for articles were performed using PubMed. The following terms were used either alone, or in combination: “synthetic glucocorticoid”, “glucocorticoid”, “betamethasone”, “dexamethasone”, “cortisol”, “prenatal stress”, “maternal stress”, “development”, “programming”, “preterm birth”, “preterm labor”, “HPA”, “fetal”, “infant”, “juvenile”, “adult”, “epigenetic”, “methylation”, “acetylation”, “miRNA”, “human”, “animal”. We focused on articles published 2010–2013, but included earlier publications when historically relevant or if the topics had not been adequately addressed in recent years. Articles selected were full-text, English-language papers. References cited in target articles were examined separately and utilized to identify additional leads.

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#### Author contributions

Both authors contributed to all aspects of the manuscript.

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