

Maternal care as a model for experience-dependent chromatin plasticity?

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It is widely acknowledged that the nature of the maternal care a child receives can have long-term repercussions, and that children raised in deprived environments can have severe cognitive and behavioural difficulties that last into adulthood. The mechanisms underlying these effects are not understood, but recent data from rodents provide insight into a potential molecular mechanism. Like humans, rodent maternal behaviour towards offspring can effect long-term changes in responses of the offspring to stress throughout the rest of their lives. Remarkably, these changes reflect permanently altered gene expression, so-called 'environmental programming', and its downstream effects on the hypothalamic-pituitary-adrenal axis. This review discusses the nature of this environmental programming – the mechanism by which it occurs in rats, its long-term implications, and opportunities for its reversal in rodents and ultimately in humans.

Introduction

The quality of early family life influences health throughout life [1]. In part, such influences appear to be mediated by effects of parent-offspring interactions on the development of neural systems, including those that mediate responses to stress [2–6]. There is evidence in humans that parental care can affect endocrine and autonomic responses to stress that endure into adulthood. Dramatic examples derive from studies on the effects of sexual and/or physical abuse on stress responses [7]. These findings represent environmental programming – instances where exposure to an environmental event in development stably alters phenotype in adulthood. A model for such programming effects lies in older studies on handling of newborn rats. Such handling decreases the magnitude of hypothalamic-pituitary-adrenal (HPA; Figure 1) responses to stress in adulthood [8–10]. Interestingly, these effects are mediated by changes in maternal care. Handling increases pup licking and grooming (LG) by the rat mother [11–13]. Predictably, adult offspring of mothers that naturally (i.e. in the absence of any experimental manipulation) show increased levels of pup LG over the

first week postpartum (high-LG mothers) show reduced plasma adrenocorticotropin hormone (ACTH) and corticosterone responses to acute stress compared with adult offspring of low-LG mothers [12,14]. These effects are, in part, mediated by the influence of maternal care on gene expression. The offspring of high-LG mothers show

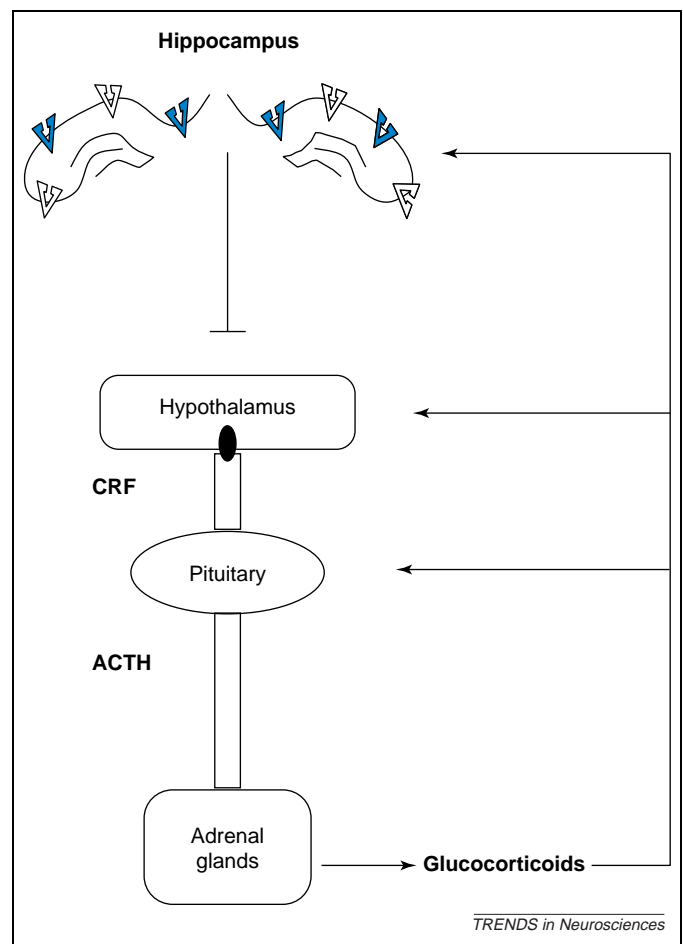


Figure 1. In response to stress, CRF, and co-secretagogues such as vasopressin, are released from paraventricular nucleus of the hypothalamus (dark oval) into the portal blood supply of the anterior pituitary, where they stimulate the synthesis and release of ACTH. Pituitary ACTH, in turn, causes release of glucocorticoids from the adrenal gland. CRF synthesis and release is subsequently inhibited through a glucocorticoid negative-feedback system mediated by mineralocorticoid and glucocorticoid receptors in several brain regions, including the hippocampus.

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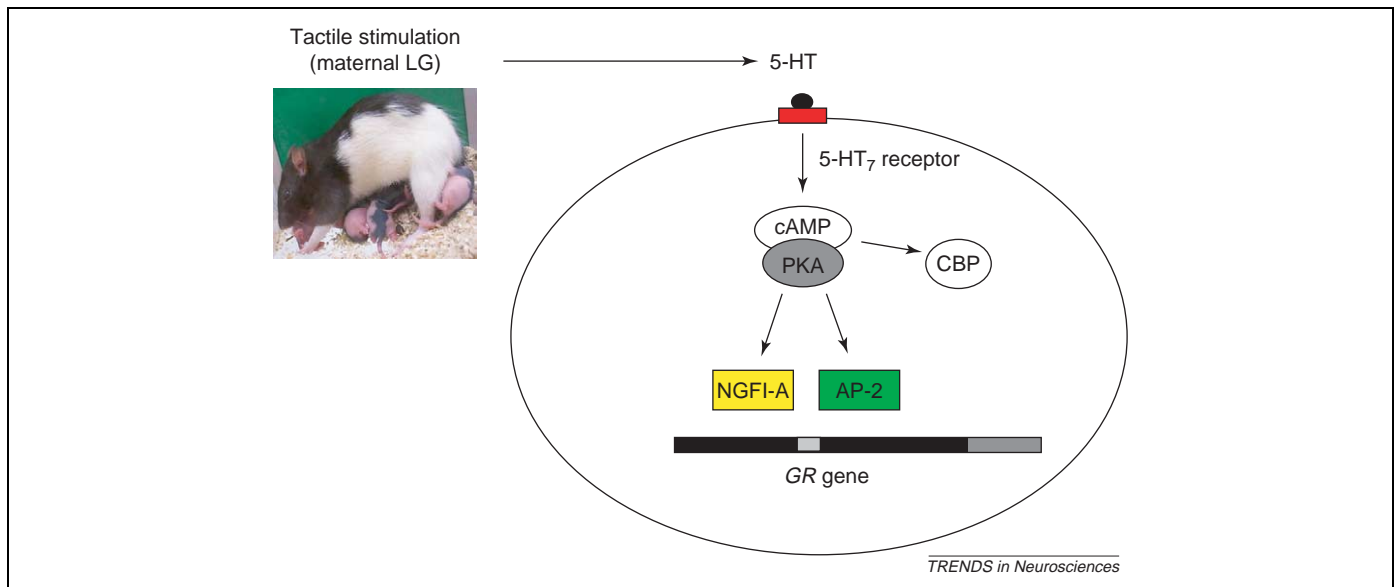


Figure 2. A summary of *in vivo* studies using hippocampal tissue from neonates and *in vitro* studies using primary hippocampal cell cultures. *In vivo*, both handling and increased maternal licking and grooming (LG) increase hippocampal 5-HT turnover. This increases activation of a 5-HT₇ receptor positively coupled to cAMP, PKA and the CREB-binding protein CBP. *In vivo*, both handling and increased maternal LG increase hippocampal cAMP concentrations and activation of PKA in pups [17–20]. Postnatal-day 6 offspring of high-LG mothers or pups of the same age exposed to handling show increased hippocampal expression of factors such as NGFI-A (also known as zif-268, krox-24, egr-1 and zenk) and the adaptor protein AP-2 [20]. *In vitro*, 5-HT increases glucocorticoid receptor (GR) expression in cultured hippocampal neurons; the effect of 5-HT on GR expression is mimicked with 8-bromo-cAMP and completely blocked by concurrent treatment using 5-HT₇ receptor antagonists [17], a PKA inhibitor or an antisense oligonucleotide directed at the NGFI-A mRNA [22]. Grey shading of the *GR* gene represents exons 1, 7 and 2–9 (see Figure 3).

significantly increased hippocampal glucocorticoid receptor (GR) mRNA and protein expression, enhanced glucocorticoid negative feedback sensitivity and decreased hypothalamic corticotrophin release factor (CRF) mRNA levels (Figure 1). Cross-fostering the biological offspring of high-LG and low-LG mothers reverses the phenotype, suggesting a direct relationship between variations in maternal care and development of HPA responses to stress [15]. Finally, reversing the effect on hippocampal GR levels eliminates the influence of early experience on HPA responses to stress [16].

Molecular mechanisms for maternal effects on HPA responses to stress

Results of *in vitro* and *in vivo* studies [10,17–21] suggest that maternal effects on hippocampal GR expression are mediated by increases in 5-hydroxytryptamine (serotonin

or 5-HT) turnover and in hippocampal expression of the transcription factor nerve-growth-factor-inducible factor A (NGFI-A, also known as zif-268, krox-24, egr-1 and zenk; Figure 2). *In vitro*, 5-HT increases expression of both GR and NGFI-A in cultured rat hippocampal neurons, and the effect of 5-HT is blocked by concurrent treatment with an antisense oligonucleotide directed to NGFI-A mRNA [22]. The 5' non-coding variable exon 1 region of the rat hippocampal *GR* gene (Figure 3) contains multiple alternate sequences, including the exon 1₇ sequence, which appears to be a brain-specific promoter [23] (see [24,25] for comparable sequences in the mouse). In adult rats, hippocampal expression of *GR* mRNA splice variants containing exon 1₇ is increased by postnatal handling [23] or maternal LG. Exon 1₇ contains an NGFI-A-binding consensus sequence (GCGGGGGCG) [26]. Maternal LG increases hippocampal NGFI-A expression, and chromatin

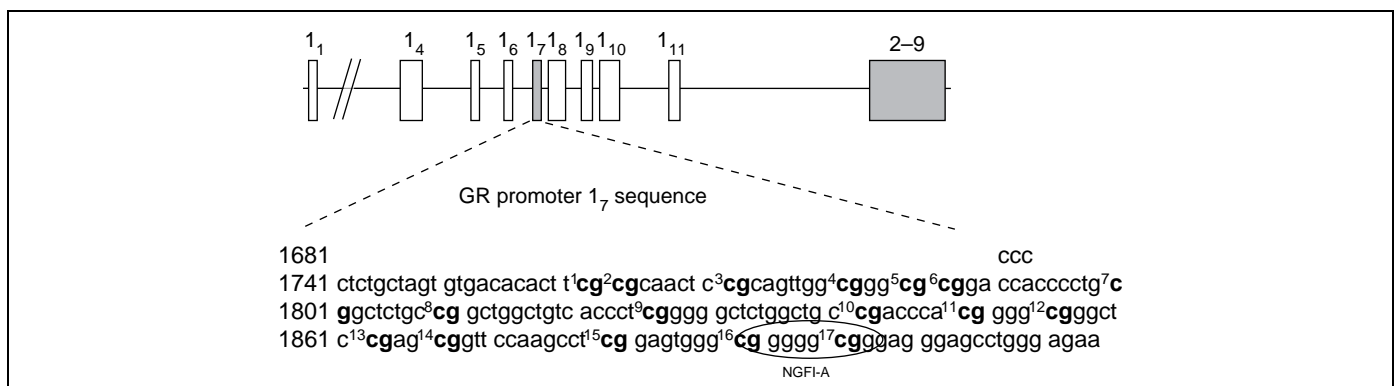


Figure 3. The 5' non-coding variable exon 1 region of the rat hippocampal glucocorticoid receptor gene contains multiple alternate exon 1 sequences [23], four of which (exons 1₁, 1₅, 1₉ and 1₁₀) correspond to alternative exon 1 sequences previously identified in mice [24,25]. Transfection studies in various cell types show that activity of constructs of the different exons fused to a luciferase reporter is similar, with one notable exception – the exon 1₇ promoter has higher activity than the other promoter constructs [23]. CpG dinucleotides in the 1₇ promoter sequence are numbered, and the NGFI-A-recognition element [26] is encircled; its 5' and 3' ends are CpG dinucleotide sites 16 and 17. Exons 2–9 represent the coding region of the *GR* gene.

immunoprecipitation (ChIP) assays with hippocampal samples from postnatal day (P)6 pups reveal that NGFI-A binding to the exon 1₇ promoter is dramatically increased in the offspring of high-LG mothers compared with the offspring of low-LG mothers (I.C.G. Weaver *et al.*, unpublished). Co-transfection of a NGFI-A vector and an exon 1₇-luciferase construct into human embryonic kidney (HEK) cells increases luciferase activity, reflecting NGFI-A-induced activation of transcription through the exon 1₇ promoter.

These findings suggest that NGFI-A might increase GR expression in hippocampal neurons and provide a mechanism for the effect of maternal care over the first week of life. However, although there are striking differences in NGFI-A expression in the neonatal offspring of high-LG and low-LG mothers, there is no effect of maternal care on hippocampal NGFI-A expression in the adult. Therefore, we asked whether the increased NGFI-A-exon 1₇ interaction in the pups of high-LG mothers might result in an epigenetic modification of the exon 1₇ sequence that alters NGFI-A binding and maintains the maternal effect into adulthood.

The epigenome: chromatin structure and DNA methylation

Most DNA is tightly packaged into nucleosomes and wrapped around a core of histone proteins [27]. The histone-DNA configuration is maintained by electrostatic bonds between positively-charged histones and negatively-charged DNA, and regulates gene expression [28]. This 'closed' chromatin structure commonly precludes transcription-factor binding to DNA and underscores the importance of enzymes that modify histone-DNA interactions. One class of such proteins, which includes many known transcriptional cofactors, are histone acetyltransferases (HATs) [29]. HATs catalyze the acetylation of selected positively-charged amino acids (e.g. lysine and arginine) on the protruding histone tails, most commonly histone (H)3 or H4. Histone acetylation of the lysine (K) residue on H3 neutralizes the positively-charged histone, opening chromatin and facilitating transcription-factor binding to DNA. Thus, H3-K9 acetylation is a marker of active gene transcription. Histone acetylation is regulated by histone deacetylases (HDACs), which block histone acetylation and suppress gene expression. Thus, intracellular signals can regulate gene expression through effects on HATs or HDACs [30-33] (or on factors involved in other histone modifications, such as methylation, phosphorylation, ribosylation and ubiquitination) that alter chromatin structure. Indeed, some environmentally regulated alterations of histone acetylation in specific promoter sequences following seizures [34,35] or learning [36] are likely to be caused by neurotransmitter activation of multiple signalling pathways [37]. However, such histone modifications are transient and cannot directly explain enduring early environmental programming effects. A more likely, highly stable candidate could involve modification of the genome itself.

DNA is covalently modified by DNA methyltransferases that transfer the methyl group from the methyl donor *S*-adenosyl methionine (SAM) to the carbon atom at

the fifth position in the cytosine ring. The carbon-carbon bond between the methyl group and cytosine residue is a stable, enduring 'epigenetic' mark. DNA methylation patterns are commonly thought to be established during early embryonic development and then faithfully maintained through life by DNA methyltransferases [30-32,38,39]. The only way methyl residues were thought to be lost was through passive demethylation (i.e. replication in the absence of DNA methyltransferase) and such processes were not considered applicable to postmitotic cells, including neurons. Methylation is involved in gene imprinting and in early phases of cellular differentiation [38]. This raises the questions of whether cellular programming through alterations in DNA methylation is unique to this period, and whether variations in methylation marks occur solely through passive demethylation.

DNA methylation promotes gene silencing through effects on chromatin structure [30-32,40-42]. In one silencing mechanism, binding of a transcription factor to its recognition element is interfered with directly, by a methylated cytosine-guanine pair (CpG) within the element - such a mechanism inhibits binding of c-Myc to its recognition element [27]. Essentially, the methylated cytosine serves as a mutation of the recognition element. A second silencing mechanism is indirect, and links DNA methylation to inactive chromatin structure. A region of methylated DNA attracts different members of a family of methylated DNA-binding proteins, such as MeCP2, which recruit HDACs [43,44] and histone methyltransferases [44] to methylated genes [32,44], blocking histone acetylation and inactivating chromatin. MeCP2 also facilitates chromatin inactivation through chromatin looping [45] and recruitment of Brahma (Brm), a catalytic component of the SWI/SNF-related chromatin-remodelling complex [46,47]. Whereas the first mechanism refers to a discrete methylation pattern, the second depends on the general density of methylated cytosine residues.

The model positioning DNA methylation as driving chromatin inactivation is well established. Nevertheless, current data suggest that chromatin structure can also determine DNA methylation, and that chromatin can affect DNA methylation in both directions, triggering either *de novo* DNA methylation [48] or demethylation [49-51]. These findings indicate that DNA methylation, although extremely stable, can be altered later in life when there is a sufficiently stable and consistent signal for chromatin activation. This relationship between chromatin state and DNA methylation forms a molecular link through which environmental signals might alter DNA methylation in specific genes in postmitotic neurons. According to this model, environmental signals trigger cellular signalling pathways, which activate *trans*-acting factors that recruit HATs, resulting in histone acetylation, chromatin opening and increased accessibility to DNA demethylating agents. This mechanism would enable reversal of the methylation mark by a similar intense change in chromatin structure later in life [52].

Epigenetic programming of HPA stress responses

We initially found evidence that methylation across the GR exon 1₇ promoter sequence in the hippocampus was

greater in adult offspring of low-LG mothers than in those of high-LG mothers [14]. These findings suggested maternal effects on DNA methylation patterns in offspring. We then examined the methylation status of individual CpGs in the exon 1₇ sequence using sodium bisulfite mapping [53], focusing on the NGFI-A consensus sequence (Figure 3). The results reveal significant differences in cytosine methylation within the 5' CpG dinucleotide of the NGFI-A consensus sequence. This site is always methylated in the offspring of low-LG mothers, and rarely methylated in those of high-LG dams. Cross-fostering reverses the differences in the methylation of the 5' CpG site and suggests a direct relationship between maternal behaviour and changes in DNA methylation within the *GR* exon 1₇ promoter [14]. The effect of maternal care is remarkably specific, with highly significant alterations in the methylation status of the 5' CpG site but no effect on the 3' site.

The difference in methylation within the 5' CpG dinucleotide of the NGFI-A-response element suggests alteration of NGFI-A binding. *In vitro*, binding of increasing concentrations of purified recombinant NGFI-A protein [54] to its response element was examined [21] using an electrophilic mobility shift assay (EMSA), with ³²P-labelled synthetic oligonucleotide sequences bearing the NGFI-A-binding site differentially methylated at the 5' or 3' CpG sites. The results indicate that methylation of the cytosine within the 5' CpG dinucleotide in the NGFI-A-response element of the *GR* exon 1₇ promoter inhibits NGFI-A binding, a finding consistent with the role for cytosine methylation.

Differences in NGFI-A expression between the offspring of high-LG and low-LG mothers in early postnatal life are no longer apparent in adulthood. Instead, it appears that the methylation of the NGFI-A consensus sequence interferes with NGFI-A binding to the *GR* exon 1₇ promoter in the offspring of low-LG mothers. Indeed, ChIP assays indicate that binding of NGFI-A protein to the hippocampal *GR* exon 1₇ promoter in adults is threefold greater in offspring of high-LG mothers than offspring of low-LG mothers [14]. Studies using the same tissue samples and an antibody against the acetylated form of H3 show increased association of acetylated H3 with the *GR* exon 1₇ promoter in offspring of the high-LG mothers. Because histone acetylation is associated with active states of gene expression, these findings are consistent with the idea of increased NGFI-A binding to the exon 1₇ promoter, and increased transcriptional activation. Moreover, transient transfection studies show that DNA methylation inhibits the ability of NGFI-A to activate the exon 1₇ promoter in HEK cells (I.C.G. Weaver *et al.*, unpublished). Taken together these findings suggest that an 'epimutation' at a single cytosine residue within the NGFI-A consensus sequence alters NGFI-A binding and might explain the sustained effect of maternal care on hippocampal GR expression and HPA responses to stress.

Reversal of maternal effects on GR expression and HPA stress responses

HDAC inhibitors can trigger active, replication-independent DNA demethylation [49,50]. We tested [14] whether

this approach could reverse the epigenetic state of *GR* in the adult offspring of low-LG mothers. Central infusion of adult rats with the HDAC-inhibitor trichostatin A (TSA) [55] significantly increased H3 acetylation, cytosine demethylation and NGFI-A binding at the exon 1₇ site in the offspring of low-LG mothers, to levels comparable with those observed in the offspring of high-LG mothers. Expression profiling of TSA-treated rats reveal specific effects of TSA on the hippocampal transcriptome (I.C.G. Weaver *et al.*, unpublished). The enhanced NGFI-A binding to the exon 1₇ promoter is associated with increased hippocampal GR expression in the offspring of low-LG mothers, to levels that are indistinguishable from those of the high-LG offspring. Most importantly, TSA infusion also eliminates the effect of maternal care on HPA responses to acute stress. Because there is considerable H3 acetylation and NGFI-A binding at the exon 1₇ sequence in the untreated offspring of high-LG mothers, TSA is without effect in these animals. These results suggest causal relationships between maternal care, histone acetylation, DNA methylation of the *GR* exon 1₇ promoter, GR expression and HPA responses to stress.

These findings, and others discussed later in this review, suggest that DNA methylation patterns are dynamic and potentially reversible even in adult neurons, which presumably bear the machinery required to demethylate genes. If DNA methylating and demethylating enzymes dynamically maintain the DNA methylation pattern in adult neurons, it should also be possible to reverse the demethylated state of the *GR* exon 1₇ promoter. Indeed, chronic central infusion of adult offspring of high-LG or low-LG mothers with the methyl-donor amino acid methionine increases DNA methylation at the NGFI-A-binding site and reduces NGFI-A binding to the exon 1₇ promoter selectively in the offspring of high-LG mothers. These effects eliminate group differences in both hippocampal GR expression and HPA responses to stress (I.C.G. Weaver *et al.*, unpublished). Methionine increases the levels of SAM and DNA methylation [56], and SAM could increase DNA methylation either by stimulating DNA methylation enzymes [57] or by inhibiting demethylases [51].

How does maternal care alter cytosine methylation?

Developmental studies provide clear evidence for an active process of 'demethylation' driven by maternal care. High-LG and low-LG mothers differ in the frequency of pup LG only during the first postnatal week [12,13,48,58]. This period corresponds to appearance of the difference in DNA methylation of the NGFI-A-response element of the *GR* exon 1₇ promoter. On embryonic-day 20 (24–28 h before birth), the entire exon 1₇ region is completely unmethylated. On P1, both the 5' and 3' CpGs of the NGFI-A site are heavily methylated in both high-LG and low-LG groups, suggesting a comparable postnatal wave of *de novo* methylation. The differences in methylation emerge between P1 and P6, which is precisely when differences in the maternal behaviour of high-LG and low-LG dams are apparent. By P6, the 5', but not the 3', CpG dinucleotide of the NGFI-A-response element is demethylated in the high-LG but not in the low-LG group, and the

maternal effect persists through to adulthood. These findings suggest that the group difference in DNA methylation involves a process of demethylation.

These data beg the question of how maternal care might activate demethylation of the exon 1₇ promoter. Note that on P1 the CpG sites of the NGFI-A consensus sequence in the exon 1₇ promoter are heavily methylated and, thus, in a 'low binding affinity' state in the offspring of both high-LG and low-LG mothers. We suggest that increased pup LG increases NGFI-A transcription by activating 5-HT₇ receptor signalling through cAMP and protein kinase A (PKA; Figure 4). Increased NGFI-A levels enhance NGFI-A binding to the methylated *GR* exon 1₇ promoter, recruitment of HATs, histone acetylation and accessibility of the *GR* exon 1₇ promoter to demethylase, resulting in DNA demethylation. The relationship between environmental stimulation, transcription-factor activation and histone acetylation has been established using hippocampal neurons [37]. However, and more controversially, this model also assumes a steady state of DNA methylation and demethylation, the direction of which is determined by the state of chromatin structure [39,40]. The hypothesis predicts that both DNA methyltransferases and demethylases are present in neurons and that alterations of the chromatin state by persistent physiological signals can alter the methylation of a gene in postmitotic tissue such as hippocampal neurons. Pharmacological activation of chromatin structure by HDAC inhibitors, such as TSA, can trigger replication-independent, active demethylation of DNA [49–51]. As already described, TSA treatment of adult offspring of low-LG mothers increases H3 acetylation and

NGFI-A binding at the exon 1₇ promoter; predictably, the treatment also results in demethylation of the 5' CpG site in the absence of cell replication [14]. In HEK cells transiently transfected using a methylated *GR* exon 1₇ promoter–luciferase vector, NGFI-A overexpression ultimately leads to demethylation of the 5' CpG in the NGFI-A-binding site within the exon 1₇ construct. Because the non-integrated plasmid does not bear an origin of replication and does not replicate in HEK cells, the assay measures the effects of NGFI-A on active, replication-independent demethylation. To demonstrate that this DNA demethylation requires direct contact between NGFI-A and its recognition element, we performed site-directed mutagenesis of the two CpGs of the NGFI-A-recognition element. Preliminary results suggest that these manipulations abolish the ability of NGFI-A to activate the *GR* exon 1₇ promoter (I.C.G. Weaver *et al.*, unpublished). Finally, hippocampal cell cultures treated with either 5-HT or 8-bromo-cAMP, a stable cAMP analogue, show increased GR expression and hypomethylation of the 5' CpG dinucleotide of the NGFI-A consensus sequence within the *GR* exon 1₇ promoter, with no effect at the 3' site (I.C.G. Weaver *et al.*, unpublished) – once again, in the absence of cell replication. Cultures maintained under control conditions show complete methylation of both the 5' and 3' CpG sites of the NGFI-A consensus sequence. Bromodeoxyuridine labelling, which marks newly generated cells, reveals little or no cell replication in the cultures at the time of 5-HT treatment; indeed, the cultures are treated with mitotic inhibitors to prevent glial proliferation. How does NGFI-A trigger demethylation upon binding to specific sequences? One possibility is that

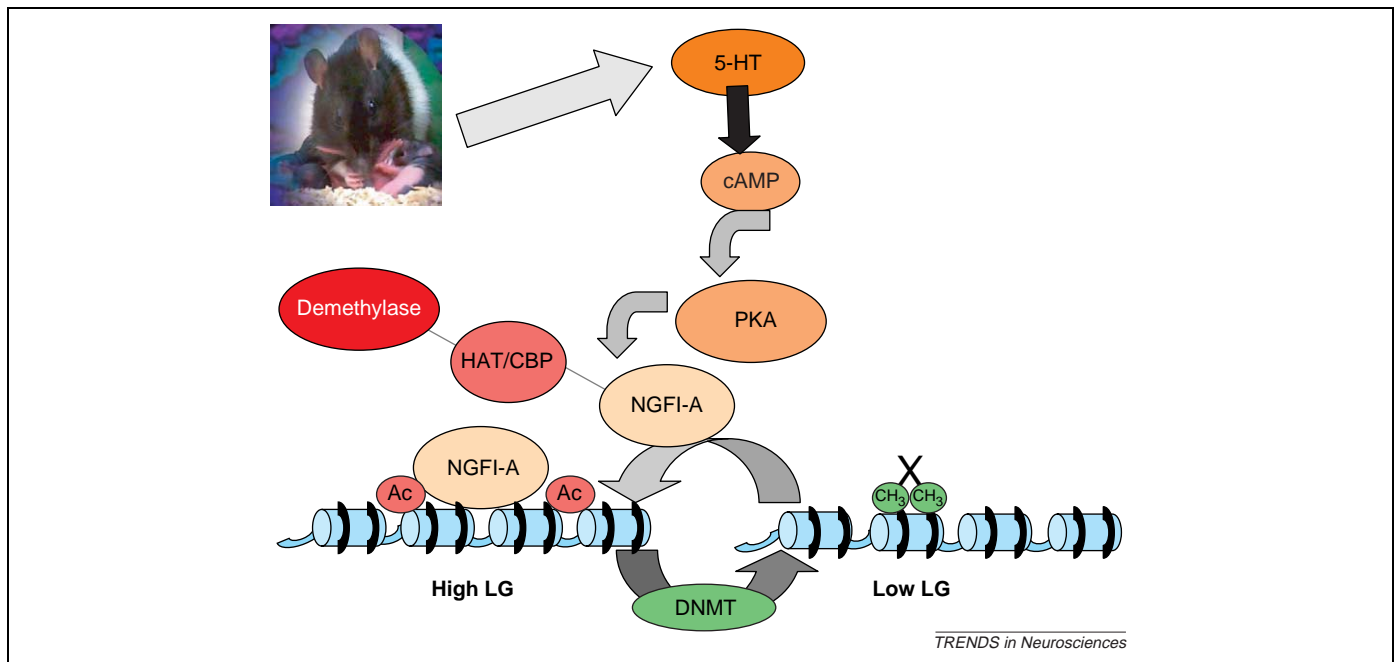


Figure 4. A proposed model for maternal programming of *GR* epigenetic states. Increased maternal LG activates NGFI-A expression (via 5-HT, cAMP and PKA; Figure 2). Although the affinity of NGFI-A to its recognition sequence on *GR* is reduced by the methylation of this element on postnatal-day 1, binding occurs through the increased levels of NGFI-A associated with enhanced tactile stimulation derived from maternal LG. Binding of NGFI-A results in recruitment of histone acetyltransferases (HATs) such as CBP; these increase histone acetylation (Ac), which in turn facilitates the access of demethylase and demethylation of the *GR* promoter, and possibly the recruitment of methylated DNA-binding proteins [59]. In the absence of increased LG and NGFI-A expression, the promoter remains methylated. The unmethylated promoter will maintain high affinity to NGFI-A throughout adulthood, resulting in greater NGFI-A-induced activity of the *GR* promoter in adult offspring of high-LG mothers, whereas the methylated *GR* promoter exhibits reduced affinity for NGFI-A, resulting in low activity of the *GR* in adult offspring of low-LG mothers. Abbreviation: DNMT, DNA methyltransferase.

NGFI-A directly recruits a demethylase to the gene; another, as already proposed here, is that NGFI-A recruits a HAT, which increases acetylation, thus increasing accessibility to demethylase. NGFI-A can actively target methylated-DNA-binding proteins to genomic targets [59]. Interestingly, 5-HT increases expression of cAMP-response-element-binding protein (CREB)-binding protein (CBP) in hippocampal cell cultures (and in *Aplysia* during conditioning [36]), and CBP binding to the *GR* exon 1₇ promoter is increased in the neonatal offspring of high-LG mothers. CBP is a HAT [60].

Conclusions: experience-dependent chromatin plasticity?

A defining question for scientists interested in development concerns the mechanism by which environmental effects, including social interactions such as parenting, in early development are 'biologically embedded' and thus sustained into adulthood. The challenge is to define the exact nature of the gene-environment interactions that mediate such environmental programming. The differential epigenetic status of the *GR* exon 1₇ promoter in the offspring of high-LG mothers is a possible mechanism for the maternal effect on hippocampal GR expression and HPA responses to stress. However, these findings are restricted to the study of a single promoter in only one gene in one brain region; at this time, these results might be best thought of as a 'proof of principle'. The degree to which such results generalize to other instances of environmental programming remains to be determined, and could reveal alternative mechanisms for programming of the epigenome.

In the context of the present model (Figure 5), further studies are required to determine precisely how maternal behaviour alters epigenomic status of the *GR* exon 1₇ promoter. The data suggest an active process of demethylation. Once considered heretical, the findings discussed here, along with a recent paper [61] on altered expression of interleukin-2 expression by T lymphocytes following activation, implicate an active process of demethylation in non-transformed somatic cells. Active demethylation now appears to be an accepted event [62]. And there is evidence for existence of a demethylase [63,64], although the exact identity of the demethylase at work in these studies remains to be clarified. Nevertheless, these findings provide a possible mechanism for the 'environmental programming' of gene expression and function during development and beyond. Studies on the reversal of maternal effects on DNA methylation using either TSA or methionine suggest that neurons express the enzymatic machinery necessary for methylation and demethylation in adulthood. DNA methylation, although a stable epigenetic mark maintained through carbon-carbon bonds, can be altered through sustained alterations of chromatin structure such as histone acetylation. These findings thus raise the fascinating question of the degree to which such processes might remain sensitive to environmental regulation throughout life.

It is important to note that maternal effects on the expression of defensive responses, such as increased HPA activity, are a common theme in biology [65]. Such effects could reflect environmental experience of the mother being translated, through an epigenetic mechanism of inheritance, into phenotypic variation in the offspring.

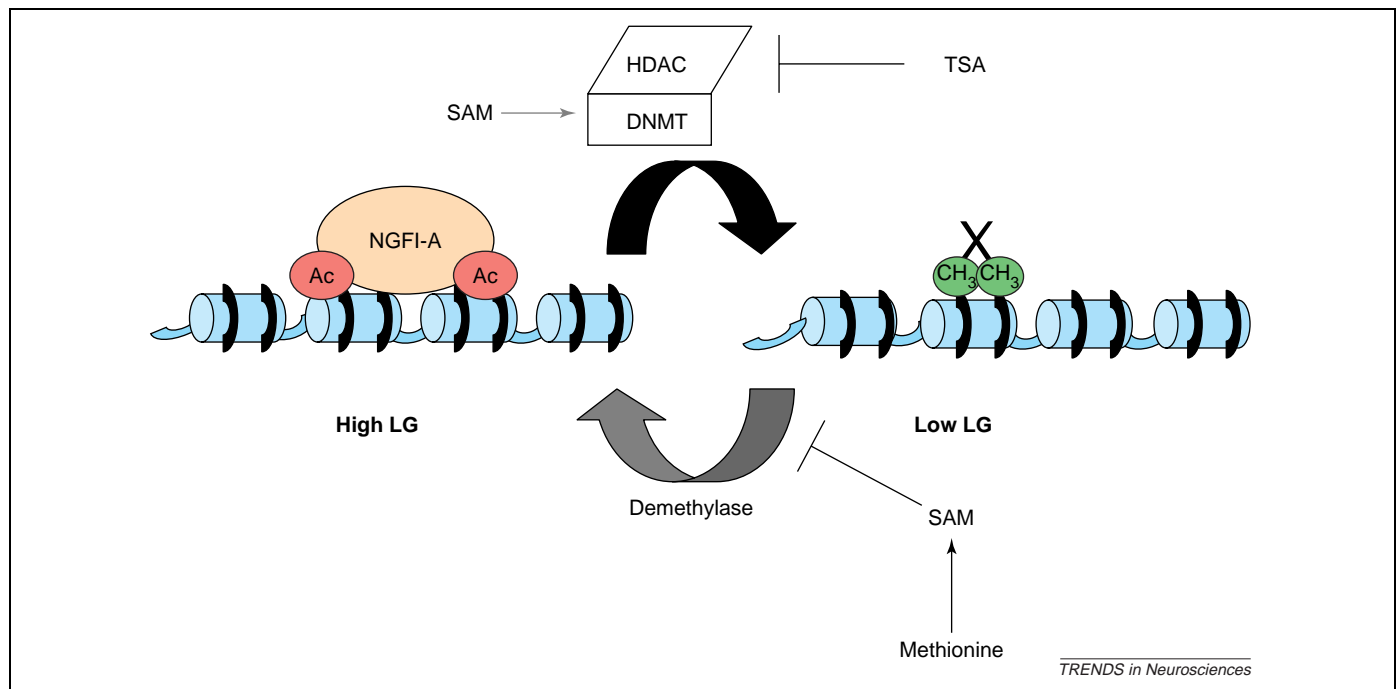


Figure 5. Reversibility of maternal-programmed GR expression and stress responses. DNA methylation patterns appear subject to a sustained modification through alterations in chromatin structure. Inhibition of histone deacetylases (HDACs) by trichostatin A (TSA) in adult animals increases histone acetylation, facilitating access of demethylase and demethylation of *GR* [14]. TSA eliminates the effect of maternal care on *GR* methylation and expression. Conversely, preliminary data reveal that central methionine treatment in adult animals increases S-adenosyl methionine (SAM) concentrations and results in the hypermethylation of *GR* and decreased GR expression, possibly through the inhibition of demethylase and the stimulation of DNA methyltransferase (DNMT). Although currently speculative, we propose that these mechanisms form the basis for environment-regulated chromatin plasticity.

Indeed, maternal effects could result in the transmission of adaptive responses across generations [10]. In humans, such effects might contribute to the familial transmission of risk and resilience. Finally, it is interesting to consider the possibility that epigenetic changes could be an intermediate process that imprints dynamic environmental experiences on the fixed genome, resulting in stable alterations in phenotype – a process of environment-dependent chromatin plasticity.

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