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Review

Environmental regulation of the neural epigenome

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A B S T R A C T

Parental effects are a major source of phenotypic plasticity. Moreover, there is evidence from studies with a wide range of species that the relevant parental signals are influenced by the quality of the parental environment. The link between the quality of the environment and the nature of the parental signal is consistent with the idea that parental effects, whether direct or indirect, might serve to influence the phenotype of the offspring in a manner that is consistent with the prevailing environmental demands. In this review we explore recent studies from the field of 'environmental epigenetics' that suggest that (1) DNA methylation states are far more variable than once thought and that, at least within specific regions of the genome, there is evidence for both demethylation and remethylation in post-mitotic cells and (2) that such remodeling of DNA methylation can occur in response to environmentally-driven, intracellular signaling pathways. Thus, studies of variation in mother–offspring interactions in rodents suggest that parental signals operate during pre- and/or post-natal life to influence the DNA methylation state at specific regions of the genome leading to sustained changes in gene expression and function. We suggest that DNA methylation is a candidate mechanism for parental effects on phenotypic variation.

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1. Introduction

Phenotypic plasticity is defined by variations in genotype–phenotype relations in response to environmental signals [1]. Such plasticity reflects the interdependence of gene and environment in defining phenotype. The focus of this paper is that of elaborating the molecular mechanisms by which environmental signals might alter the structure and the function of the genome to produce what are statistically represented in gene × environment interaction effects in genetics [2–4].

The challenges in understanding the biology of phenotypic plasticity refer to both ultimate (function and evolutionary origin) and proximal causes. In this paper we emphasize proximal causation, focusing on the identity of the relevant environmental signals and defining the underlying biological mechanisms with respect to genome function and specific phenotypic outcomes. We consider in particular parental influences, which are a major source of phenotypic plasticity. We examine the hypothesis that the parental programming of gene transcription and broader measures of phenotype reflected in such parental effects is mediated by epigenetic mechanisms that stably alter gene transcription and thus physiology and behavior [5–7].

2. Parental effects

Parental effects are defined as sustained influences on any component of the phenotype of the offspring that derives from a parental signal, apart from nuclear genes; an influence of parental phenotype on that of the offspring (e.g. [8,9]). There is evidence for parental effects on multiple phenotypic outcomes, especially on growth, reproductive tactics and defensive responses. Phenotypic plasticity is also recognized by developmental psychobiologists as the product of gene × environment interactions occurring throughout development [2,3,10,11], with variations in parent–offspring interactions serving as the relevant environmental signal.

Environmental signals operate during development to stably affect (i.e., "program") gene transcription [6]. Environmental programming of gene transcription provides an ideal candidate mechanism for the phenotypic plasticity implied by life history theory [12]. The question is that of how environmental signals occurring at specific times in development might program the

Abbreviations: CRF, corticotropin-releasing factor; HPA, hypothalamic–pituitary–adrenal axis; LG, licking/grooming; PVNh, paraventricular nucleus of the hypothalamus; GABA, gamma aminobutryic acid; ERa, estrogen receptor alpha; 5-HT, serotonin; NGFI-A, nerve-growth factor-inducible factor-A; T3, triidodithyronine; ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; H, histone; HDAC, histone deacetylase; SAM, s-adenosyl methionine; TSA, trichostatin A; AVP, arginine vasopressin; MeCP2, methyl CpG-binding protein 2; PPAR-α, peroxisomal proliferator-activated receptor alpha; POMC, proopiomelanocortin; CBP, CREB binding protein

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operation of the genome and, in particular, how such effects endure into adulthood – well beyond the period of exposure to the critical inducing agent. It is this latter feature that defines an 'environmental programming' effect.

Parental effects are apparent across a variety of species ranging literally from plants to mammals [8,9]. Such effects are likely due to the fact that natural selection has the shaped offspring to respond to subtle variations in parental signals as a forecast of the environmental conditions they will ultimately face following independence from the parent [9,13]. Apart from environmental stability across generations, what is critical for hypotheses proposing that parental effects enhance the match between the phenotype of the offspring and the demands of the environment is whether the nature of the parental signal that produces the phenotypic outcome is systematically associated with both (1) the quality of the prevailing environment and (2) a specific developmental outcome. Is the parental signal a reliable basis for prediction of environmental quality and does it systematically alter phenotypic development in the appropriate direction?

Some of the most interesting examples of phenotypic plasticity derive from instances in which the development of the animal is shaped by environmental misfortune, most commonly in the form of poor nutrient supply or high rates of predation. We define a developmentally "adverse" environment as one in which an animal is constantly required to divert energy from growth to defensive systems (i.e., a shift from anabolism to catabolism) in the interest of meeting an immediate environmental threat. Studies in avian species provide evidence for a systematic relation between the quality of the environment at the time of reproduction and nature of the relevant parental signal. The parental signal often takes the form of yolk steroid hormone levels that vary as a function of the quality of the maternal environment [14]. Maternal transfer of steroid hormones to the yolk thus provides a parental signal that links the quality of the environment to the phenotypic development of the offspring. It is important to note that such effects can often be considered as adaptive. Thus, while increased exposure to maternal corticosterone signals may constrain growth [6,14], there is also an increase in the flight performance of fledglings associated with expanded pectoral muscle mass and wing area [15]. Maternal corticosterone levels reliably track environmental adversity, including the risk of predation as well as nutrient availability. Increased flight performance enhances the chances for escape from predation, and is important for animals occupying a niche with an increased risk of capture. An enhanced flight performance in the offspring should improve predator avoidance. This is but one example of parental effects that appear to serve not only predator escape, but also to increase the probability of successful reproduction or foraging under conditions of adversity [8,9,16,17]. In this instance a more stressful environment has launched a series of trade-offs that are reflected in stable phenotypic variation.

3. Parental effects on stress responses

Parental signaling will vary in a species-specific manner. Nutrient provision is a common parental signal in insects (e.g. [18]) and yolk steroids in birds [14]. Both signaling systems likely operate in mammals [6,19] along with the enhanced potential for postnatal influences through active parental care. Research with non-human primates and rodents support a causal link between the quality of the environment, parent–offspring interactions and phenotypic development in mammals. These studies reveal evidence for direct effects of stress on the quality mother–infant interactions with subsequent effects on phenotypic development. For example, in Bonnet macaques, restricted access to food is an obvious stressor for lactating females and impairs the quality of mother–infant interaction reflected by an increase in the rate of maternal rejection, which associates with increased fearfulness and stress reactivity in the offspring [20]. The more stressful foraging conditions affect the development of neural systems that mediate behavioral and endocrine response to stress in the offspring. Adult monkeys reared under such conditions showed increased central levels of corticotropin-releasing factor (CRF) and increased noradrenergic responses to stress (Fig. 1).

Likewise, in the rat and mouse pup licking/grooming (LG) by the mother, which actively stimulates an anabolic endocrine state [21,22], is diminished by chronic stress [23–25]. Variations in pup LG in the rat directly affect the development of behavioral and hypothalamic–pituitary–adrenal (HPA) responses to stress in adulthood [25–33]. Rat mothers exhibit considerable naturally-occurring variations in the frequency of pup LG over the first week

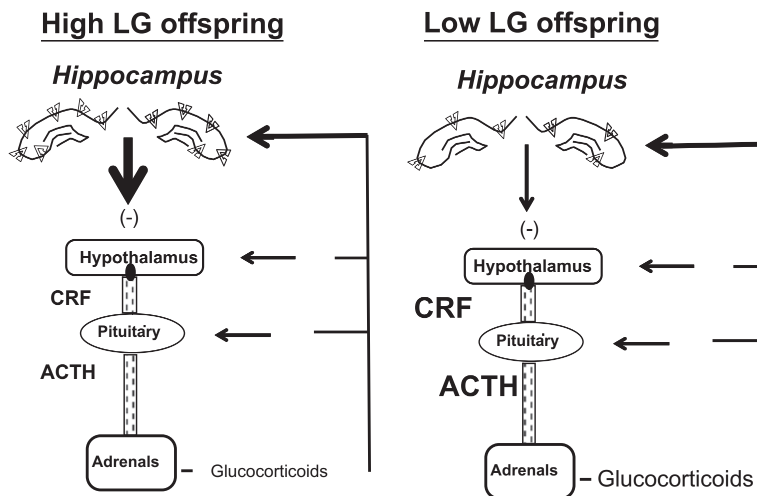

Fig. 1. A schema outlining the function of the hypothalamic–pituitary–adrenal axis, the nexus of which are the corticotropin-releasing factor (CRF) neurons of the paraventricular nucleus of the hypothalamus. CRF is released into the portal system of the anterior pituitary stimulating the synthesis and release of adrenocorticotropin (ACTH), which then stimulates adrenal glucocorticoid release. Glucocorticoids act on glucocorticoid receptors in multiple brain regions, including the hippocampus, to inhibit the synthesis and release of CRF (i.e., glucocorticoid negative feedback). The adult offspring of High licking/grooming (LG) mothers, by comparison to those of Low LG dams, show (1) increased glucocorticoid receptor expression, (2) enhanced negative-feedback sensitivity to glucocorticoids, (3) reduced CRF expression in the hypothalamus, and (4) more modest pituitary–adrenal responses to stress (references provided in text).

of life, even within the confines of an animal vivarium [34,35]. These individual differences in maternal behavior are stable across litters, such that the frequency of pup LG for the first litter strongly predicts that for the second, third and even fourth litters, assuming that environmental conditions remain stable. Chronic stress over gestation or an impoverished environment during peripubertal development decreases the frequency of pup LG in the female rat [23–25,36].

Maternal licking associates with individual differences in the HPA response to stress in adulthood. The response to stress at the level of the pituitary is governed by the release of CRF (Fig. 1). CRF stimulates the synthesis and release of adrenocorticotropin (ACTH) from the pituitary, which in turn, stimulates the release of adrenal glucocorticoids (principally corticosterone in rodents and cortisol in primates). As adults, the offspring of High LG mothers show more modest plasma ACTH and corticosterone responses to acute stress by comparison to animals reared by Low LG mothers [26,30,31]. Circulating glucocorticoids act at glucocorticoid receptor (GR) sites in corticolimbic structures, such as the hippocampus, to regulate HPA activity (Fig. 1). Such feedback effects target CRF synthesis and release at the level of the paraventricular nucleus of the hypothalamus (PVNh). The offspring of High LG mothers show significantly increased hippocampal GR mRNA and protein expression, enhanced glucocorticoid negative feedback sensitivity and decreased hypothalamic CRF mRNA levels. Intra-hippocampal infusion of a GR antagonist eliminates the maternal effect on HPA responses to stress, suggesting a direct relation between hippocampal glucocorticoid receptor expression and the magnitude of the HPA response to stress.

Variations in maternal care in the rat are sensitive to the quality of the pre- and post-natal environment, with downstream effects on developmental outcomes. Thus, maternal stress reduces the frequency of pup LG and decreases hippocampal GR expression in the offspring [23]; such effects are directly mediated by the decrease in postnatal pup LG. Manipulations, such as the brief handling of pups by the experimenter, that increase the frequency of pup LG by the mother increase hippocampal glucocorticoid receptor expression, decrease that of CRF in the hypothalamus and dampen HPA responses to stress [28,33,37]. Finally, the frequency of pup LG is also influenced by light cycles that mimic the daylight associated with seasonal change [33]. Shorter periods of 'daylight' are associated with decreased pup LG and accompanied by changes in hippocampal GR expression and HPA responses to stress in adulthood. Pup LG provides tactile stimulation. Artificial forms of tactile stimulation, such as brushing pups, increases hippocampal GR expression [38] and dampen HPA responses to stress [39]. Thus, environmental conditions act during postnatal life to program HPA responses to stress through effects on maternal care mediated by tactile stimulation.

4. Molecular transduction of parental signals

Variations in maternal care associate with sustained alterations in GR expression and the regulation of HPA responses to stress. The defining feature of the maternal programming effect is the sustained changes in gene expression that endure beyond the period of life that involves maternal care and which subserve phenotypic variation at the level of behavior and physiology. The results of the cross-fostering studies as well as those using experimental tactile stimulation suggest that the findings described above reveal direct effects of postnatal maternal care and for the tactile stimulation that derives from pup LG. Tactile stimulation derived from maternal licking appears to be the critical signal for the regulation of hippocampal GR expression and HPA responses to stress. Indeed, *within-litter* variation in the frequency with which individual pups are licked is significantly correlated with hippocampal GR mRNA levels in adulthood [40]. Finally, artificial tactile stimulation of rat pups, which mimics that afforded by licking, increases hippocampal GR expression [38].

The results of in vivo studies with tissue samples from rat pups or in vitro studies using cultured primary hippocampal neurons suggest that maternal effects on GR expression are mediated by increases in hippocampal serotonin (5-HT) activity and the expression of the transcription factor, nerve-growth factor-inducible factor-A (NGFI-A) [41–46]. In vitro, 5-HT increases the activity of cAMP-dependent signaling pathways in hippocampal neurons through the activation of a 5-HT_7 receptor resulting in elevated expression of the transcription factor, NGFI-A. Activation of this signaling cascade leads to increased GR expression. The effect of 5-HT on GR expression in cultured hippocampal neurons is (1) blocked by 5-HT_7 receptor antagonists or compounds that inhibit the activation of protein kinase A, (2) mimicked by 5-HT_7 receptor agonists or treatments with stable cAMP analogs (e.g., 8-bromo-cAMP), and (3) eliminated by concurrent treatment with an anti-sense directed at the NGFI-A mRNA [45,46]. In vivo, the increase in hippocampal 5-HT activity is associated with a maternally-regulated increase in the conversion of thyroxine to triidodithyronine (T3) [46]. T3 regulates the activity of ascending 5-HT systems and neonatal administration of T3 mimics the effects of increased pup LG on hippocampal GR expression [47]. In vivo, T3 administration increases hippocampal NGFI-A expression [48] and this effect as well as that on GR expression are blocked with 5-HT receptor antagonists [46] (Hellstrom and Meaney, unpublished).

The DNA site at which maternally regulated, 5-HT-induced NGFI-A signal alters GR expression involves distinct regions of the 5′ non-coding variable exon 1 region of the hippocampal glucocorticoid receptor gene (Fig. 2). This region contains multiple alternate promoter sequences including the exon 1_7 sequence, which is highly expressed in brain [49]. Increased levels of pup LG enhance hippocampal expression of GR mRNA splice variants containing exon 1_7 [45], suggesting greater transcriptional activity through this promoter. The exon 1_7 sequence contains an NGFI-A response element [49,50]. Pup LG increases hippocampal NGFI-A expression and chromatin immunoprecipitation (ChIP) assays, which permit quantification of protein interactions with specific DNA sequences, with hippocampal samples reveal increased NGFI-A association with the exon 1_7 promoter in pups of High compared with Low LG mothers [45]. Co-transfection studies reveal NGFI-A-induced activation of transcription through the exon 1_7 promoter [45]. The effect of NGFI-A is eliminated by a site-directed mutation within the NGFI-A response element of the exon 1_7 promoter [45] such that the physical interaction of NGFI-A with its response element triggers transcriptional activation. Infection of hippocampal neurons with an NGFI-A expression plasmid increases both total GR mRNA and exon 1_7-containing GR mRNA [46].

These findings suggest that NGFI-A increases GR expression in hippocampal neurons through the exon 1_7 promoter and provide a mechanism for the effect of maternal care over the first week of life. The results of subsequent studies suggest that the increased NGFI-A–exon 1_7 interaction occurring within hippocampal neurons in the pups of High LG mothers might result in an epigenetic modification of the exon 1_7 sequence that alters NGFI-A binding and maintains the maternal effect into adulthood.

5. The epigenome: chromatin structure and DNA methylation

Epigenetics refers a set of biochemical signals that directly or indirectly alter genomic structure and function (i.e., transcription) without an alteration in nucleotide sequence [51,52]. The study of epigenetics focuses on the relation chromatin structure and gene

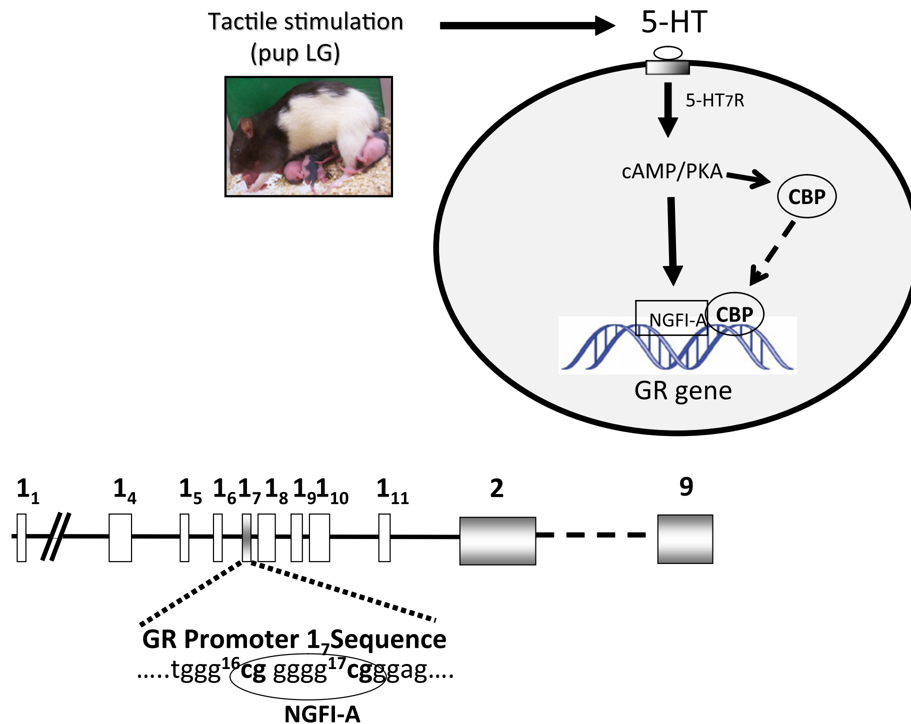

Fig. 2. (Top) A summary of in vivo studies with hippocampal tissue samples from neonates and in vitro studies using primary hippocampal cell cultures. In vivo, maternal LG increases hippocampal 5-HT turnover, activation of a 5-HT_7 receptor positively coupled to cAMP and cyclic nucleotide dependent kinases (PKA). The day 6 offspring of High LG mothers show increased hippocampal expression of NGFI-A (also referred to as zif-268, krox-24, and egr-1). 5-HT increases both glucocorticoid receptor expression in cultured hippocampal neurons; the effect of 5-HT on GR expression is mimicked with 8-bromo-cAMP and blocked by concurrent treatment 5-HT_7 receptor antagonists, a PKA inhibitor or an oligonucleotide antisense or shRNA directed at the NGFI-A mRNA. (Bottom) A schema describing the organization of the rat glucocorticoid receptor gene including 9 exon regions. Exons 2–9 code for the glucocorticoid receptor protein. Exon 1 is comprised of multiple promoter sequences that can independently initiate transcription. The various exon 1 promoters actions are tissue-specific, with evidence suggesting that certain promoters are more active in areas such as liver or thymus, and others more active in brain (e.g., exon 1_7; based on Ref. [49]). The nucleotide sequence of the exon 1_7 promoter is described below, highlighting the NGFI-A response element.

transcription. DNA is commonly packaged into nucleosomes and wrapped tightly around a core of histone (H) proteins [53–56]. Chemical modifications that regulate chromatin structure influence transcriptional activity, in part, through effects on transcription factor binding.

The classical epigenetic mark on DNA is that of methylation, which in mammals occurs uniquely at cytosines. Cytosine methylation at sites that lie within promoter/enhancer regions is often associated with gene silencing [54,51,57–59]. Such effects occur either through the direct interference with transcription factor binding or indirectly through mediators that favor a closed chromatin structure that reduces the probability of transcription factor binding to DNA. Such indirect effects are mediated by the binding of methylated DNA binding proteins to methylated DNA and the associated recruitment of repressor complexes that includes histone deacetylases (HDACs) [54,60]. HDAC's limit histone acetylation, thus promoting an inactive chromatin state. DNA methylation is implicated in cases where highly stable programming of gene expression is established in early life, such as gene imprinting, X-chromosone inactivation, and in early phases of cellular differentiation [61]. Studies in cancer biology suggest that the loss of methylation associates with pathology [62–64]. However, studies of the epigenetic reprogramming of the paternal genome in early embryonic development suggest active demethylation [61] (and see [65,66]), as do more recent research with specific regions of the genome, such as the promoter for the *IL2* gene [67,68]. Likewise, studies of astrogliogenesis in the forebrain [69–72] suggest a considerable capacity for 'plasticity' in cytosine methylation, revealing a condition that is dynamic and subject to both *active* demethylation and remethylation in the post-mitotic state. These studies reveal that states of DNA methylation in post-mitotic cells are subject to modification by environmental signals. Thus, within at least certain regions of the genome, DNA methylation states appear to be dynamic and responsive to environmentally-driven alterations in intra-cellular signaling activity.

DNA methylation can favor inactive chromatin states. Nevertheless, the relation between DNA methylation and gene expression includes discussion of whether methyl marks and chromatin structure are necessarily a cause or consequence of changes in gene expression [56,73]. The disruption to the nucleosome structure that accompanies transcriptional activity necessitates a 're-packaging' of chromatin following the cessation of gene transcription [56]. The form of the re-packaging, including potential re-modeling of epigenetic marks, could influence the probability of subsequent transcriptional activity. There is evidence that alterations to chromatin structure can re-define DNA methylation states, and that chromatin alterations can affect both de novo DNA methylation or demethylation [74–77]. Increasing histone acetylation leads to transcription factor binding at previously methylated sites, and to demethylation. HDAC inhibitors act in synergy with 5-aza-2-deoxycytidine to demethylate DNA and increase transcriptional activation. Overexpression of the transcription factor SP-1 associates with both an increase and a decrease in the level of cytosine methylation, depending upon the pre-existing level of methylation [78,79]. DNA methylation, although stable, is altered in response to the opening of chromatin structure and transcriptional activation. During gliogenesis increased binding of Stat3 to the *gfap* promoter increases transcriptional activity and site-specific demethylation (e.g. [69–72]). Calcium-dependent depolarization of hippocampal neurons leads to an increase in the transcription of the BDNF gene that associates with a decrease in the methylation of the exon IV promoter [80] (and see [81] for a review). BDNF is associated with

synaptic remodeling. Environmentally-dependent neural functions such as learning and memory, that require synaptic remodeling, associate with alterations in the methylation states of genes, the products of which are integral for learning [82].

The relation between chromatin state (and transcription factor binding) and DNA methylation forms a molecular link through which environmental signals might potentially re-organize DNA methylation marks in specific genes in post-mitotic cells. Accordingly, environmental signals trigger cellular signaling pathways that activate trans-acting factors that recruit chromatin remodeling complexes and increase accessibility to DNA demethylating/methylating agents. Such a process could allow for a reversal of the methylation mark by transcription factor signaling. Indeed, there is recent evidence [83,84] that both demethylation and remethylation might occur over remarkable short periods of time, reflecting a process that is, at least at certain regions of the genome, highly dynamic and closely linked to transcriptional regulation. In one exciting model [84] DNA methylation/demethylation of the cytochrome p450 27B1 (*CYP27B1*) gene promoter is dynamically regulated by vitamin-D-mediated transrepression, which associates with increased methylation, and parathyroid hormone-induced activation, which associates with demethylation. Parathyroid hormone induced PKC-dependent phosphorylation of MBD-4 promotes demethylation through an MBD4-dependent DNA glycosylation/DNA repair mechanism. The dynamic nature of epigenetic signals and the possibility that even the more stable epigenetic marks, such as DNA methylation, might be altered by environmentally-sensitive intracellular signaling pathways positions epigenetics as an ideal candidate mechanism for the phenotypic plasticity that characterizes parental effects. These findings, together with those described below, form the basis of what we might term the 'environmental epigenetics' hypothesis, to wit environmental events activate intracellular signals that remodel the epigenome, leading to sustained alterations in the structure and function of the genome, and thus stable effects on gene transcription.

6. The epigenetics of phenotypic plasticity

Epigenetic modifications are a candidate mechanism for the effects of maternal care on hippocampal GR expression and HPA responses to stress. Studies using sodium bisulfate mapping to examine the methylation status of individual CpGs in the exon 1_7 sequence reveal significant differences in cytosine methylation at the 5′ CpG dinucleotide of the NGFI-A consensus sequence. This site is hypermethylated in the offspring Low LG mothers, and hypomethylated in those of High LG dams. Cross-fostering reverses the differences in the methylation of the 5′ CpG site and suggests a direct relation between maternal care and DNA methylation of the exon 1_7 GR promoter [30]. The effect of maternal care is specific, with significant alterations in the methylation status of the 5′ CpG, and no effect at the 3′ site. Nevertheless, although less striking, there are differences in the frequency of methylation at other CpG sites on the exon 1_7 promoter [30,86]. Indeed, studies using methylation-dependent DNA precipitation with high coverage tiling arrays across chromosome 18 in the rat, which includes the GR gene, reveal broad regions that differ in cytosine methylation [87].

In vitro binding of purified recombinant NGFI-A protein to its response element indicate that methylation of the cytosine of the 5′ CpG dinucleotide in the NGFI-A response element of the exon 1_7 GR promoter inhibits NGFI-A protein binding [45]. The methylation of the NGFI-A consensus sequence also associates with decreased in vivo NGFI-A binding to the GR exon 1_7 promoter in the offspring of Low LG mothers [30,31,45]. ChIP assays indicate a three-fold greater binding of NGFI-A protein to the exon 1_7 GR promoter in hippocampal samples obtained from the adult offspring of High compared with Low LG mothers. Importantly, such differences occur despite a comparable level of hippocampal NGFI-A expression in the adult offspring of High and Low LG mothers. The methylation of the 5′ CpG site appears to functionally alter the 'affinity' of the NGFI-A consensus sequence for its ligand, resulting in decreased NGFI-A binding.

Studies with the same tissue samples and an antibody against the acetylated form of H3 show increased acetylated lysine 9 (H3-K9) associated with the exon 1_7 GR promoter in the offspring of the High LG mothers [30,31]. Since H3-K9 acetylation associates with active states of gene expression, these findings support the idea of increased NGFI-A binding to the exon 1_7 promoter, and increased transcriptional activation. Moreover, transient transfection studies provide evidence that (1) NGFI-A induces transcription through the exon 1_7 promoter and (2) DNA methylation of a transfected exon 1_7 promoter construct inhibits the ability of NGFI-A to bind to and activate expression through the exon 1_7 promoter [45]. Taken together these findings suggest that variation in the methylation status of the exon 1_7 sequence alters NGFI-A binding and might explain the sustained effect of maternal care on hippocampal GR expression and HPA responses to stress. Finally, the sequencing involved in these studies has yet to reveal any evidence for sequence variation in this region. Thus, to the best of our knowledge, the individual differences in GR expression in this model associates with variation at the level of epigenetic state, and not in nucleotide sequence. This finding occurs despite the fact that sequence information can affect epigenetic state [88,89].

The effect of CpG methylation on gene expression is, in part, mediated by the binding of methylated DNA binding proteins and the recruitment of repressor complexes that include HDACs. HDAC inhibitors permit chromatin remodeling and transcription factor binding, and may thus liberate the expression of genes from methylation-induced repression. HDAC inhibition indeed reverses the maternal effects on hippocampal GR expression [30]. Chronic, central infusion of adult offspring of Low LG mothers with the broad spectrum HDAC inhibitor, trichostatin A (TSA), significantly increased both H3-K9 acetylation of and NGFI-A binding to the exon 1_7 promoter in the offspring of Low LG mothers to levels comparable to those observed in the offspring of High LG mothers. The enhanced NGFI-A binding to the exon 1_7 promoter is associated with increased hippocampal GR expression in the offspring of Low LG mothers to levels indistinguishable from those of the offspring of High LG mothers, and the elimination of the effect of maternal care on HPA responses to acute stress. These results suggest a direct relation between maternal care, the epigenetic state of the exon 1_7 GR promoter, GR expression and HPA responses to stress.

The findings from studies of hippocampal GR gene expression parallel those of Murgatroyd et al. [90] who examined the effects of prolonged periods of maternal separation on the development of HPA responses to stress in the mouse. Maternal separation in rodents increases the magnitude of HPA responses to acute stress (e.g. [91]). Increased HPA activity associated with maternal separation is accompanied by a persistent increase in arginine vasopressin (AVP) expression in neurons of the hypothalamic paraventricular nucleus and is reversed by an AVP receptor antagonist [90]. AVP acts in synergy with CRF to increase pituitary ACTH synthesis and HPA activity (Fig. 1). The altered *Avp* expression associates with sustained DNA hypomethylation of a regulatory region containing CpG residues that serve as DNA-binding sites for the methyl CpG-binding protein 2 (MeCP2). MeCP-2 binding regulates *avp* expression. As in the case with GR regulation, *avp* expression correlates with the methylation status of a single CpG site. A rather unique analysis showed that the difference in the methyla-

tion of this CpG remained stable from 3 to 12 months of age, reflecting the potential stability of early environmental effects.

These studies suggest that social experience can dynamically influence DNA methylation states in post-mitotic neurons that associate with stable changes in gene expression. Beyond the examples cited above, variations in maternal care are also associated with alterations in the methylation state of the promoter for the estrogen receptor alpha (ERa) gene [92] as well as that for BDNF [25]. Such effects are not limited to the environmental conditions of early life. Chronic stress in mice produces a sustained demethylation of the CRF gene [93]. Such conditions commonly associate with increased CRF expression and enhanced fearfulness. Interestingly, in this study *CRF* demethylation is apparent only in those animals that subsequently reveal stress-induced social fear.

A set of recent studies [94] provides at least correlational evidence for the idea that comparable epigenetic modifications might occur in humans in response to variations in parent–offspring interactions. These studies examined the methylation status of the exon 1_F promoter of the GR gene, which corresponds to the exon 1_7 promoter in the rat [95], in hippocampal samples obtained from victims of suicide or controls (sudden, involuntary death). There is increased DNA methylation of the exon 1_F promoter in hippocampal samples from suicide victims compared with controls, but only if suicide was accompanied by a developmental history of child maltreatment (physical or sexual abuse, or persistent neglect). Child maltreatment, independent of psychiatric state or concurrent drug use, predicted the DNA methylation status of the exon 1_F promoter. Moreover, the methylation state of the exon 1_F promoter determined NGFI-A binding to the promoter and transcriptional activation. Such studies are correlational and limited by post-mortem approaches. Nevertheless, the results are consistent with the hypothesis that variations in parental care can modify the human epigenome. The findings are also consistent with studies that link childhood abuse to individual differences in CRF activity [96] and HPA stress responses [97]. Childhood abuse associates with an increase in pituitary ACTH responses to stress among individuals with or without concurrent major depression. The ACTH findings are particularly relevant since pituitary ACTH directly reflects central activation of the HPA stress response and hippocampal GR activation dampens HPA activity. These findings are consistent with the rodent studies cited above suggesting that epigenetic mechanisms can mediate environmentally-induced, stable variations in phenotype.

Parental effects are also apparent in studies examining the influence of perinatal nutritional states on metabolic function. The parent is the major proximal source of variation in nutrient supply, and such variations stably alter the expression of genes implicated in glucose and lipid metabolism, as well as in insulin sensitivity [6,19,98]. Such effects include hepatic expression of regulators of lipid and glucose metabolism such as peroxisomal proliferator-activated receptor alpha (PPAR-α) and GR. There is evidence that these changes in gene expression are mediated by epigenetic mechanisms. A rat model of in utero protein restriction produces increased hepatic PPAR-α and GR gene expression and associates with hypomethylation of the respective promoters [99]. These findings follow from the pioneering studies showing that dietary supplements directly alter DNA methylation [7,100,101]. Perinatal nutritional signals also promote adult obesity through effects on feeding and energy expenditure [98]. Studies using postnatal overnutrition suggest that perinatal programming of ‘appetite’ occurs as a function of epigenetic regulation of gene expression. Rats reared in small litters (overfed during suckling) show increased methylation of the proopiomelanocortin (POMC) gene promoter at the NF-κB binding site compared with controls [102]. Importantly, these animals show a decreased anorexic response to leptin (i.e., central leptin resistance) that correlates with the hypermethylation of the PMOC promoter. Leptin constrains food intake through, in part, an increase in POMC expression. Nutritional signals, including those of maternal origin, clearly hold a remarkable potential for remodeling the neural epigenome, especially within brain regions that govern feeding behavior, energy expenditure and metabolism.

7. How do parental signals alter DNA methylation?

Environmental signals, including those of parental origin, influence DNA methylation at specific regions of the genome. One question concerns the processes by which such environmental signals might directly alter epigenetic marks, including CpG methylation. Developmental time course studies of the methylation state of the exon 1_7 GR promoter provide evidence for an *active and targeted process* of “demethylation” driven by intracellular signals associated with the tactile stimulation derived from pup LG. We summarize below the evidence for this aspect of the environmental-epigenetics hypothesis. The research to date suggests that environmentally-regulated signals can lead to stable alterations of the epigenome. However, we note that there are serious gaps in our knowledge, particularly concerning the identity of the relevant catalytic enzymes. This is more particularly true for studies suggesting active demethylation.

High and Low LG mothers differ in the frequency of pup LG only during the first week of life [27,34]. This period corresponds to the appearance of the difference in DNA methylation of the NGFI-A response element of the exon 1_7 GR promoter [30]. On embryonic day 20 (24–28 h prior to birth) the entire exon 1_7 region is completely unmethylated. However, by postnatal day 1 both the 5′ and 3′ CpGs of the NGFI-A site are heavily methylated regardless of maternal phenotype suggesting a comparable postnatal wave of de novo methylation. The differences in the methylation of the exon 1_7 GR emerge between postnatal days 1 and 6, which corresponds to the period when differences in the pup LG between High and Low LG mothers are apparent. By day 6 of postnatal life, the 5′, but not the 3′, CpG dinucleotide of the NGFI-A response element is demethylated in the offspring of High, but not in the Low LG group. This maternal effect persists through to adulthood.

Pup LG increases NGFI-A transcription as a result of activation of a 5-HT_7 receptor signaling through cAMP and protein kinase A. In pups treated with the 5-HT toxin, parachlorylamphetamine (PCA), maternal licking has no effect on NGFI-A association with the exon 1_7 GR promoter (Hellstorn and Meaney, unpublished). The activation of PKA is coupled to an increase in the expression of the CREB binding protein (CBP) and there is increased CBP binding to the exon 1_7 GR promoter in the neonatal offspring of High LG mothers [45]. Thus, 5-HT increases CBP expression in hippocampal cell cultures [41,103]. CBP is a histone acetyltransferase (HAT) that associates directly with NGFI-A (104). The proteins appear to bind as a complex at the exon 1_7 promoter site in response to maternal licking. Thus, the site-directed mutation within the exon 1_7 promoter that blocks the binding of NGFI-A to its response element, also reduces CBP association at this site [45]. Since CBP is a HAT, increased association with the exon 1_7 promoter could result in an opening of chromatin, despite the hypermethylation of the site at postnatal day 1, and increase access of NGFI-A to its response element.

There is preliminary evidence (Hellstrom and Meaney, unpublished) directly links the increased association of NGFI-A to the exon 1_7 GR promoter to mother–pup interactions. In such studies tissue samples obtained immediately following a nursing bout, during which time the mother is on the nest and actively interacting with her pups (i.e., the “ON” condition), or after 25 min without mother–pup contact (a normal interlude between nursing bouts;

i.e., the mother "OFF" condition). The results of ChIP assays show increased association of both NGFI-A and CBP with the exon 1_7 GR promoter in the offspring of High compared to Low LG mothers. However, the difference is observed only in hippocampal samples obtained following the ON condition. Moreover, artificial tactile stimulation derived from simply stroking the pups with a brush is sufficient to increase NGFI-A association with the exon 1_7 promoter. These findings are consistent with an earlier report showing that the same 'stroking' of pups increases hippocampal GR expression [38] and dampens HPA responses to stress [39]. These findings suggest that it is the tactile stimulation associated with pup LG that is critical for the effect on hippocampal NGFI-A association with the exon 1_7 GR promoter.

The critical issue is whether the maternally-regulated hippocampal signals, including the actual binding of NGFI-A to the exon 1_7 sequence, directly alter the methylation status on the exon 1_7 promoter. Hippocampal cell cultures treated with either 5-HT or 8-bromo-cAMP, a stable cAMP analog, show increased GR expression [41–45] and hypomethylation of the 5′ CpG dinucleotide of the NGFI-A consensus sequence within the exon 1_7 GR promoter [45]. As in the in vivo condition, there is no effect at the 3′ site. Cultures maintained under control conditions show hypermethylation of both the 5′ and 3′ CpG sites. The effects on DNA methylation occur in the absence of cell replication. Bromo-deoxyuridine labeling, which marks newly generated cells, reveals little or no cell replication in the cultures at the time of 5-HT treatment and indeed the cultures used in these studies are treated with mitotic inhibitors to prevent glial proliferation. The loss of the methyl signal is therefore not explained by a passive demethylation (i.e., the loss of a methyl mark in the course of cell replication). Moreover, the effect of 5-HT on either GR expression or the methylation status of the affected 5′ CpG site is blocked when cells are treated with an antisense directed against NGFi-A mRNA [45].

The studies with primary hippocampal neurons suggest that specific intracellular signals initiate an NGFI-A-dependent remodeling of DNA methylation at the NGFI-A response element. As discussed above intrahippocampal TSA treatment of adult offspring of Low LG mothers increases H3-K9 acetylation and NGFI-A binding at the exon 1_7 promoter in the offspring of Low LG mothers (30). This condition mimics the signals observed at the exon 1_7 promoter site in the neonate in response to pup LG. Predictably the TSA-induced increase in NGFI-A binding to the exon 1_7 promoter is associated with a *demethylation* of the 5′ CpG site [30,45].

The overexpression of NGFI-A, over time, leads to increased NGFI-A binding and enhanced transcriptional activity in HEK cell transfected with a vector bearing a construct that includes an methylated glucocorticoid receptor exon 1_7 promoter-luciferase construct [45]. Methylation of the exon 1_7 sequence reduces both NGFI-A binding as well as NGFI-A-induced transcriptional activity. However, although significantly reduced by comparison to the unmethylated construct, NGFI-A overexpression does result in NGFI-A association with the methylated exon 1_7 sequence, and ultimately leads to significant demethylation of the 5′ CpG site in the NGFI-A response element [45]. Interestingly, site-directed mutagenesis of the 3′ CpG within the NGFI-A response element completely abolishes the ability of NGFI-A to interact with the NGFI-A response element. Studies with this construct show no effect of NGFI-A overexpression on the methylation status of the 5′ CpG site. Thus, overexpression of NGFI-A can alter the methylation state of the 5′ CpG site, and this effect is dependent upon the interaction between NGFI-A and it's response element. Interestingly, the same pattern emerges in study of another NGFI-A-sensitive target, glutamic acid decarboxylase 1 (GAD1), which encodes for the GAD_{67} protein. Maternal licking increases NGFI-A association with the GAD1 promoter in the offspring [105]. Predictably, the adult offspring of High LG mothers show decreased methylation of the GAD1 promoter that associates with increased H3-K9ac and NGFI-A binding, and enhanced GAD1 expression. Moreover, hippocampal neuronal cultures treated with 5-HT also show increased GAD1 NGFI-A occupancy of the GAD1 promoter and increased GAD1 expression, suggesting a common signaling pathway.

GAD1 has been the focus of pioneering studies of DNA methylation and neural dysfunction. Cortical dysfunction in schizophrenia is associated with changes in gamma aminobutryic acid (GABA) ergic circuitry [106] accompanied by a decrease in *GAD1* expression [107,108] as well as in reelin, which is closely associated with synaptic plasticity. The GABAergic neurons in the schizophrenic brain that express reelin and GAD67 exhibit an increase in DNA methyltransferases 1 (DNMT1) [109]. The promoter for the *reelin* gene shows increased methylation in the brains of patients with schizophrenia compared with control subjects [110,111]. The inhibition of DNMT1 in neuronal cell lines increases the expression of both reelin and GAD1, that associates with a decreased association of MeCP2, potentially linking the alteration in DNA methylation to the decrease in expression [112]. Likewise the effect of maternal care is associated with a decrease in DNMT1 expression and reduced MeCP2 association with the *GAD1* promoter. The studies of Grayson and colleagues suggest that methylation states in fully differentiated neurons is actively maintained and dynamically regulated through the regulation of DNMT1 expression.

The results of the TSA study described above suggest that DNA methylation patterns are dynamic, even in adult animals. Moreover, intrahippocampal infusion of the methyl donor amino acid methionine [113] leads to a remodeling of the methylation state at the 5′ CpG site of the NGFI-A response element, in this case producing hypermethylation of the site in the adult offspring of High LG animals. Thus, chronic central infusion of adult offspring of High or Low LG mothers with methionine increases DNA methylation at the NGFI-A binding site and reduces NGFI-A binding to the exon 1_7 promoter sequence selectively in the offspring of High LG mothers. These effects eliminate group differences in both hippocampal GR expression and HPA responses to stress [113]. Methionine increases the levels of s-adenosyl methionine (SAM) and DNA methylation. The obvious implication of such studies, as well as those with GAD1 and reelin, is that fully differentiated cells expression the enzymatic machinery necessary for remodeling DNA methylation through active methylation and demethylation. The caveat is that the active demethylase in each of these instances has yet to be identified.

8. Conclusions

Multiple recent reviews summarize the compelling evidence for the environmental regulation of histone methylation states [114,115]. The research to date is also generally consistent with the 'environmental epigenetics' hypothesis, suggesting that environmentally-induced alteration in cell signaling pathways actively remodel DNA methylation states. These findings are also consistent with those of in vitro models, revealing stunningly dynamic regulation of DNA methylation [67,68,82–84]. Although there are a limited range of research models in which this issue has been directly addressed, the evidence in rodents is consistent with the idea that nutritional signals as well as variations in parent–offspring interactions can influence the methylation state of specific genes, which then mediate parental effects on phenotype. In addition to the results of such studies, we suggest that the features of DNA methylation, which to some extent have only recently come to be appreciated, are consistent with those of a mechanism for parental effects. Ironically, the dynamic nature and environmental sensitivity of DNA methylation in fully differentiated cells is somewhat at odds with the stability that is considered as a strength for the can-

didacy of DNA methylation as a mechanism for parental effects [73]. Programming would seem to imply a transient period of plasticity, followed by unwavering stability. In truth we know rather little about the variation in methylation marks at specific loci over time within the same individual (but see [90]). One possibility is that once induced, the epigenetic mark is maintained through environmental signals that are, in some way, consistent with those during early development. One possible example is that of insulin resistance and obesity. Developmental studies of metabolic 'programming' suggest that insulin resistance may appear in early development individuals born small for gestational age. Insulin resistance can promote obesity, which in turn, could sustain the state of insulin resistance. Whether or not such a process could operate at the level of the epigenome is completely speculative, but is consistent with the 'potentiation' models advanced to explain the ying/yang of chromatin remodeling and gene transcription [52,56,73], whereby transcriptional activation associates with chromatin states that enhance the probability of subsequent transcriptional activity, providing a feed-forward loop. Additionally the potential for tissue-specific alterations of DNA methylation, suggested by the environmental epigenetics hypothesis, is a critical feature for the enduring effects such as parental influences, learning or repeated exposure to drugs of abuse [82,115]. The environmental alteration of methylation marks in post-mitotic cells provides an opportunity for tissue specific effects on DNA methylation. Tissue specificity in methylation states might emerge as a natural consequence of environmentally-regulated effects on cell-specific, intracellular signaling pathways. The fact that fully differentiated cells express the enzymatic machinery necessary for remodeling methylation states and that the expression of methylated DNA binding proteins as well as DNA methyltransferases is influenced by environmental signals provides the basis for environmentally-driven, tissue specific effects on DNA methylation. The range of genomic regions subject to such influences remains a question that has yet to be addressed, awaiting genome-wide analyses.

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