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Review

Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat

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Abstract

Increased levels of pup licking/grooming and arched-back nursing by rat mothers over the first week of life alter the epigenome at a glucocorticoid receptor gene promoter in the hippocampus of the offspring. Differences in the DNA methylation pattern between the offspring of High and Low licking/grooming—arched-back mothers emerge over the first week of life, are reversed with cross-fostering, persist into adulthood and are associated with altered histone acetylation and transcription factor (NGFI-A) binding to the glucocorticoid receptor promoter. Central infusion of the adult offspring with the histone deacetylase inhibitor trichostatin A removes the previously defined epigenomic group differences in histone acetylation, DNA methylation, NGFI-A binding, glucocorticoid receptor expression, and hypothalamic-pituitary-adrenal responses to stress, thus suggesting a causal relation between the epigenomic state, glucocorticoid receptor expression and the effects of maternal care on stress responses in the offspring. These findings demonstrate that an epigenomic state of a gene can be established through a behavioral mode of programming and that in spite of the inherent stability of this epigenomic mark, it is dynamic and potentially reversible.

Keywords: Maternal behavior; Glucocorticoid receptor; Stress responses; DNA methylation; Gene expression; Histone acetylation; NGFI-A; Estrogen receptor

1. Introduction

The quality of early family life influences the risk for multiple forms of chronic illness over the lifespan [173]. While these effects are clearly apparent in the risk for mental illness [9,16,22,24,69,84,85,87,142,153,209,215,223], such factors also predict visceral obesity, type II diabetes, coronary heart disease as well as gastroenterological and obstetric outcomes [39,48,54,68,102,108,117,163,164,181,215]. 'Stress diathesis' models (Fig. 1) have emerged as explanations for the relation between the quality of early life and health in adulthood. These models suggest that adversity in early life alters the development of neural and endocrine responses to stress and thus predisposes individuals to disease in adulthood (e.g. [75,76,82,126,129,163,187,198]). The relation between the quality of the early environment and health in adulthood appears to be mediated by parental influences on the development of neural systems that underlie the expression of behavioral and endocrine responses to stress [59,75,76,140,187,198]. Indeed, in human and nonhuman primates adversity or decreased quality of parental investment increases the magnitude of emotional, autonomic, central catecholamine and hypothalamic-pituitary-adrenal (HPA) responses to stress in adulthood [7,75,76,82,112,166,175,203]. In nonhuman primate and rodent models, repeated and prolonged periods of maternal separation over the first weeks postpartum result in enhanced behavioral and HPA responses to stress [7,60,13,20,43,82,109,129,160,162].

The logic for stress diathesis models is buttressed by the strong evidence for the endangering effects of prolonged

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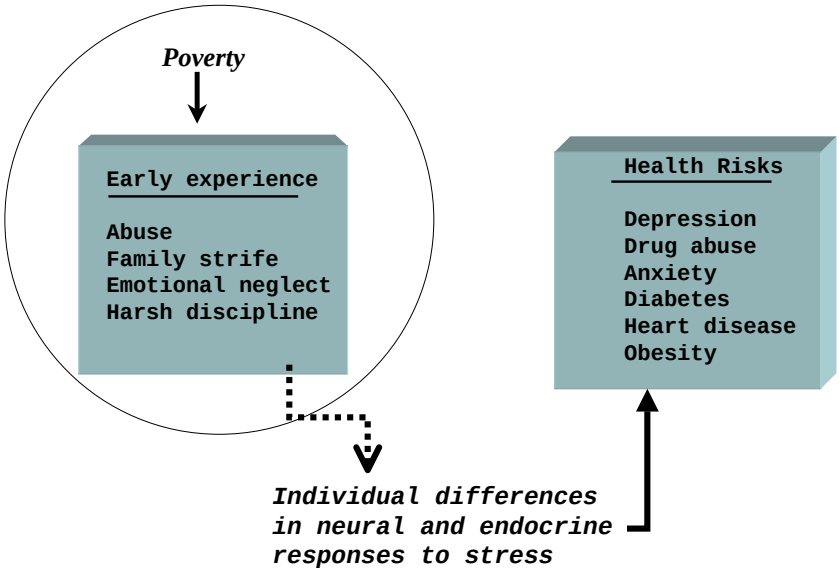

Fig. 1. A schema outlining the pathways implied in stress diathesis models. Familial adversity, such as poverty, influences the nature of parent–child interactions, which in turn influence the development of individual differences in neural and endocrine responses to stress. Resulting variations in exposure to stress mediators, such as corticotropin-releasing hormone (CRF), catecholamines and glucocorticoids, can then serve as the basis for individual differences in the risk for multiple forms of chromic-illness. According to such models the critical conditions are the presence in early life of forms of parent–offspring interactions that promote increased stress responses and chronic stress in adulthood. The focus of this chapter is the proposed link between variations in parent–offspring interactions and the development of individual differences in stress responses.

exposure to 'stress hormones' that provides further support for stress diathesis models. Thus, chronic exposure to elevated levels of stress hormones, including corticotrophin-releasing factor (CRF), catecholamines, most notably norepinepherine, and glucocorticoids promote the development of a diverse range of high risk conditions, such as visceral obesity, hypertension and insulin intolerance, or overt pathology, including diabetes, depression, drug addiction and multiple forms of coronary heart disease [34,37,38,119,157,177,184,212]. Clinical-case studies show that increased cortisol levels are associated with visceral obesity, cardiovascular disease, type II diabetes and depression (for a review see [174]). Moreover, in prospective epidemiological studies, measures of peripheral catecholamines and glucocorticoids predict an increased risk for cardiovascular disease and overall mortality [189,190]. Moreover, polymorphisms of the glucocorticoid receptor are associated with chronic cardiovascular and metabolic illness [214]. The clinical risks associated with prolonged activation of the HPA and autonomic systems are a logical consequence of the otherwise adaptive and highly catabolic stress response. The increased release of glucocorticoids from the adrenal gland and catecholamines, particularly norepinepherine from the sympathetic system, increases the availability of energy substrates, such as those derived from lipids and glucose metabolism, in order to maintain the normal cellular output and organ efficiency. These actions protect against catastrophes such as hypotensive shock. These hormones, along with the central CRF, act on multiple brain regions to increase vigilance and fear, and enhance avoidance learning and fear conditioning, which reduces the chances of further encounters with the offending conditions. Prolonged exposure to CRF, glucocorticoids and catecholamines drives hyperlipidemia, increased cardiovascular tone, insulin resistance, and alterations of mood.

Support for the basic elements stress diathesis models appears compelling. Adversity during perinatal life alters development in a manner that seems likely to promote vulnerability, especially for stress-related diseases. Diathesis describes the interaction between development, including the potential influence of genetic factors, and the prevailing level of stress in predicting health outcomes. Such models have considerable appeal, and could potentially identify both the origins and the nature of vulnerability derived from either epigenetic influences, such as early family life, or genomic variations. The question of interest to our labs is that of how environmental events in early life, particularly those involving parent–offspring interaction, might regulate the development of individual differences in stress responses. Of particular interest is the question of how early experience produces sustained effects on phenotype.

2. The development of individual differences in stress responses

The studies of Levine, Denenberg and others reveal that in rodents, postnatal handling (aka, infantile stimulation) alters the development of responses to adverse stimuli, or stressors [44,103,104]. The handling paradigm involves a brief, daily (i.e., ~15 min) separation of the pups from the dam. This period falls well within the range normal mother–pup separations that lie between nursing bouts and does not seem to constitute any major deprivation of

parental care. In infant rats and mice, handling during infancy decreases the magnitude of both behavioral and (HPA) responses to stress in adulthood. These findings clearly demonstrate that the early environment influences the development of even rudimentary defensive responses to threat. These were landmark data in an era in which the development of such basic features of phenotype were rather blithely described as "innate" (a term for which the frequency of use was matched only by the paucity of any useful and testable definition).

The weakness of the handling model was that it was entirely artificial. However, Levine and others suggested that the effects of handling on defensive responses were actually mediated by changes in maternal care. The idea was that handling pups affected the behavior of the mother towards her offspring and that such effects were critical for the influences on pup development. Indeed handling increases pup licking/grooming by the mother [101,110]. More recent studies support the maternal-mediation hypothesis. One approach is to examine the consequences of naturally occurring variations in pup licking/grooming across unmanipulated dams [28]. These studies indicate that the adult offspring of mothers that naturally show a high level of pup licking/grooming and nurse more frequently in the arched-back nursing (ABN) posture (i.e., High LG-ABN mothers; Fig. 2) resemble postnatally-handled animals on measures of behavioral and endocrine responses to stress, while those of Low LG-ABN mothers are comparable to nonhandled animals [19]. Note, of course, that the phenotype of the nonhandled pups typically varies considerably since such animals are randomly derived from High, Mid and Low LG-ABN mothers. Specifically, the adult offspring of High LG-ABN mothers show decreased fearfulness under conditions of mild stress and more modest HPA responses to stress as measured by plasma levels of either ACTH or corticosterone [19,58,110,127,217]. Cross-fostering studies, where pups born to High LG-ABN mothers are fostered at birth to Low LG-ABN mothers (and vice versa), suggest a direct relationship between maternal care and the postnatal development of individual differences in behavioral and HPA responses to stress [20,58,217]. Finally, these studies suggest that variations within a normal range of parental care can dramatically alter development. In large measure this is likely due to the fact that natural selection has shaped offspring to respond to subtle variations in parental behaviors as a forecast of the environmental conditions they will ultimately face following independence from the parent ([83] and see Section 7). A critical element of this argument is the potential adaptive importance of altered HPA responses to stress depending upon the prevailing environmental demands. This issue is considered in the final section of this paper.

2.1. Maternal care in the rat: Behavioral and HPA responses to stress

Central CRF systems furnish the critical signal for the activation of behavioral, emotional, autonomic and endocrine responses to stressors. There are two major CRF pathways regulating the expression of these stress responses. First, a CRF pathway from the parvocellular regions of the paraventricular nucleus of the hypothalamus to the hypophysial-portal system of the anterior pituitary, which serves as the principal mechanism for the transduction of a neural signal into a pituitary-adrenal response [3,79,159,176,221]. In responses to stressors, CRF, as well as co-secretagogues such as vasopressin, are released from hypothalamic neurons into the portal blood supply of the anterior pituitary where they stimulate the synthesis and

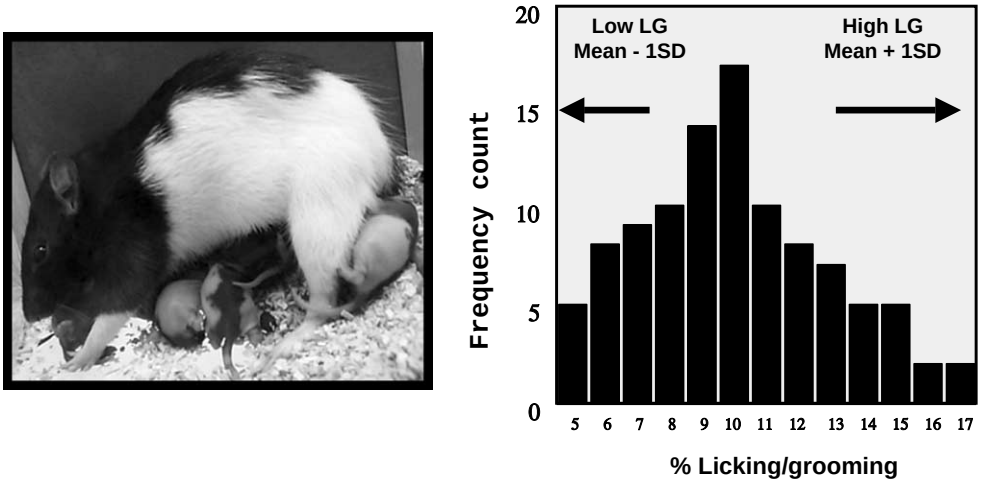

Fig. 2. (Left) A photograph of a nursing Long-Evans mother and her pups. The mother is nursing in the arched-back nursing (ABN) posture while licking/grooming a pup. (Right) A frequency histogram (adapted from [28,29]) showing the distribution of pup licking/grooming (LG) scores over the first week postpartum in a large sample of lactating Long-Evans female rats. Mothers whose scores are 1 SD or more above or below the mean are deemed High LG-ABN dams; those 1 SD or more below the mean are designated Low LG-ABN mothers. *Note*. There are no differences in the total contact time with pups, litter size, birth weights, or weaning weights between High and Low LG-ABN mothers (reviewed in [28,29]).

release of adrenocorticotropin hormone (ACTH). Pituitary ACTH, in turn, causes the release of glucocorticoids from the adrenal gland. CRF synthesis and release is subsequently inhibited through a glucocorticoid negative-feedback system mediated by both mineralocorticoid and glucocorticoid receptors in a number of brain regions including the hippocampus [12,42,184]. Importantly variations in cellular levels of glucocorticoid receptors define glucocorticoid sensitivity (see [138] for a review) and a forebrain-specific glucocorticoid receptor knockout results in impaired glucocorticoid negative feedback regulation of the HPA axis and HPA hyperactivity [11a].

CRF neurons in the central nucleus of the amygdala project directly to the locus coeruleus and increase the firing rate of locus coeruleus neurons, resulting in increased noradrenaline release in the vast terminal fields of this ascending noradrenergic system. Thus, icv infusion of CRF increases extracellular noradrenaline levels [49,151,211]. The amygdaloid CRF projection to the locus coeruleus [70,93,134,211,213] is also critical for the expression of behavioral responses to stress [4,18,40,96,107,186,200,204]. Hence, the CRF neurons in the hypothalamus and the central nucleus of the amygdala serve as important mediators of both behavioral and endocrine responses to stress.

The critical question concerns the potential consequences of the differences in behavior for the development of behavioral and neuroendocrine responses to stress. As adults, the offspring of High LG-ABN mothers show reduced plasma ACTH and corticosterone responses to acute stress by comparison to the adult offspring of Low LG-ABN mothers [110,217]. Circulating glucocorticoids act at glucocorticoid and mineralocorticoid receptor sites in corticolimbic structures, such as the hippocampus, to regulate HPA activity [42,184]. Such feedback effects commonly target CRF synthesis and release at the level of the hypothalamus. The High LG-ABN offspring show significantly increased hippocampal glucocorticoid receptor mRNA expression, enhanced glucocorticoid negative feedback sensitivity and decreased hypothalamic CRF mRNA levels. Moreover, Liu et al. [110] found that the magnitude of the corticosterone response to acute stress was significantly correlated with the frequency of both maternal LG ($r = -0.61$) and ABN ($r = -0.64$) during the first week of life, as was the level of hippocampal glucocorticoid receptor mRNA and hypothalamic CRF mRNA expression (all r's > 0.70).

The offspring of the High and Low LG-ABN mothers also differ in behavioral responses to stress [19,58,127]. As adults, the offspring of the High LG-ABN show decreased startle responses, increased open-field exploration, and shorter latencies to eat food provided in a novel environment. The offspring of Low LG-ABN mothers also show greater burying in the defensive burying paradigm [127], which involves an active response to a threat. The offspring of the High LG-ABN mothers exhibit increased $GABA_A$/benzodiazepine receptor levels in the basolateral and central nucleus of the amygdala, as well as in the locus coeruleus [19,20]. A recent study reveals increased benzodiazepine sensitivity in the offspring of the High LG-ABN mothers [63]. Benzodiazepine agonists suppress CRF expression in the amygdala [146] and predictably, there is decreased CRF mRNA in the central n. of the amygdala in the adult offspring of High LG-ABN dams [Francis, Diorio, Meaney, unpublished].

A series of in situ hybridization studies [20] illustrate the molecular mechanism for the differences in $GABA_A$/benzodiazepine receptor binding and suggest that variations in maternal care might actually permanently alter the subunit composition of the $GABA_A$ receptor complex in the offspring. The offspring of the High LG-ABN mothers show increased levels of the mRNAs for the $\gamma 1$ and $\gamma 2$ sub-units, which contribute to the formation of a functional benzodiazepine binding site. Such differences are not unique to the γ sub-units. Levels of mRNA for the $\alpha 1$ sub-unit of the $GABA_A$/benzodiazepine receptor complex are significantly higher in the amygdala and locus coeruleus of High compared with Low LG-ABN offspring. The $\alpha 1$ sub-unit appears to confer higher affinity for GABA, providing the most efficient form of the $GABA_A$ receptor complex, through increased receptor affinity for GABA.

The differences in the amygdala of both $GABA_A$/BZ receptor binding and CRF expression as a function of maternal care are of potential importance for HPA function. As discussed above, CRF projections from the amygdala enhance the release of noradrenaline from catecholaminergic neurons in the locus coeruleus and the n. tractus solitarius. Moreover, the adult offspring of High LG-ABN mothers also show increased $\alpha 2$ noradrenergic receptor binding in the locus coeruleus [19]. Such $\alpha 2$ sites serve as inhibitory autoreceptors. Not surprisingly, the adult offspring of Low LG-ABN mothers show increased noradrenaline release in the paraventricular n. of the hypothalamus by comparison to those of High LG-ABN dams. Since noradrenaline acts at the PVNh as a major source of drive over CRF synthesis and release [161], alterations in CRF activity within the amygdala–locus coeruleus complex are likely to affect HPA responses to stress.

The results of these studies suggest that the behavior of the mother towards her offspring can 'program' behavioral and neuroendocrine responses to stress in adulthood. Neural systems that regulate CRF synthesis and/or release appear to be primary targets for such maternal effects, and would seem to be ideal mechanisms for maternal influences on stress reactivity. These effects are associated with sustained changes in the expression of genes in brain regions that mediate responses to stress, and form the basis for stable individual differences in stress reactivity. It is important to note the tissue specificity for such effects. To the best of our current knowledge, maternal care affects glucocorticoid receptor expression only in the hippocampus and perhaps prefrontal cortex [Zhang, Meaney, unpublished], while effects on the $GABA_A$ receptor subunits are largely confined to the amygdala and locus coeruleus; no such effects are observed in the thalamus, hippocampus,

septum or cortex. Effects on the α2 noradrenergic receptor are apparent only in the locus coeruleus.

2.2. Cross-fostering studies: Evidence for direct maternal effects

Individual differences in behavioral and neuroendocrine responses to stress in the rat are associated with naturally occurring variations in maternal care. Such effects might serve as a nongenomic mechanism by which selected traits are transmitted from one generation to another. Indeed, Low LG-ABN mothers are more fearful in response to stress than are High LG-ABN dams [57]. Hence, fearful mothers beget more stress reactive offspring. The obvious question is whether the transmission of these traits occurs only as a function of genomic-based inheritance. If this is the case, then the differences in maternal behavior may simply be an epiphenomenon, and not causally related to the development of individual differences in stress responses. The issue is not one of inheritance, but the mode of inheritance.

Reciprocal cross fostering of the offspring of Low and High LG-ABN mothers provide direct evidence for a nongenomic transmission of individual differences in stress reactivity and maternal behavior [58]. The primary concern with such studies is that the wholesale fostering of litters between mothers is known to affect maternal behavior [114]. To avert this problem and maintain the original character of the host litter, no more than 2 of 12 pups were fostered into or from any one litter [116]. The critical groups of interest are the biological offspring of Low LG-ABN mothers fostered onto High LG-ABN dams, and vice versa. The limited cross-fostering design did not result in any effect on group differences in maternal behavior. Hence, the frequency of pup licking/grooming and arched-back nursing across all groups of High LG-ABN mothers was significantly higher than that for any of the Low LG-ABN dams regardless of litter composition.

The results of these studies are consistent with the idea that variations in maternal care are causally related to individual differences in the behavior of the offspring. The biological offspring of Low LG-ABN dams reared by High LG-ABN mothers were significantly less fearful under conditions of novelty than were the offspring reared by Low LG-ABN mothers, including the biological offspring of High LG-ABN mothers [58]. Subsequent studies reveal similar findings for hippocampal glucocorticoid receptor expression and for the differences in both the α1 and γ2 $GABA_A$ receptor subunit expression in the amygdala [20]. These findings suggest that individual differences in patterns of gene expression and behavior can be directly linked to maternal care over the first week of life.

3. Environmental programming of glucocorticoid receptor expression

Both postnatal handling, which increases maternal licking/grooming, and increased levels of licking/grooming produce elevations in 5-HT turnover in the hippocampus in rat pups [131,196]. Interestingly, postnatal handling results in specific increases in 5-HT in the hippocampus and prefrontal cortex, where glucocorticoid receptor expression is selectively increased [122,196]. Serotonin levels in the hypothalamus, septum and amygdala are unaffected, and in these regions glucocorticoid receptor expression is not altered by handling.

Subsequent studies suggest that 5-HT directly alters glucocorticoid receptor expression in hippocampal neurons, an effect that is consistent with the results of in vivo studies showing that selective ablation of ascending 5-HT projections decreases glucocorticoid receptor expression in the hippocampus [188]. In vitro, the treatment of primary hippocampal cell cultures with 5-HT increases glucocorticoid receptor expression [50,81,98,99,130,132] and this effect is mediated by 5-HT_7 receptor activation [98]. The 5-HT_7 receptor is positively coupled to cAMP and glucocorticoid receptor expression in cultured hippocampal neurons is significantly increased after treatment with 8-bromo cAMP or with various doses of the specific 5-HT_7 receptor agonist, 3-(2-Aminoethyl)-1H-indole-5-carboxamide maleate (5-carboxamidotryptamine; 5-CT) for four days. This time course resembles that for 5-HT. The effect of 5-CT on glucocorticoid receptor expression is blocked by methiothepin [98]. Likewise, 5-CT produces a significant increase in cAMP levels and the effect is blocked by methiothepin. Pindolol, which binds to the 5-HT_{1A} but not the 5-HT_7 receptor, has little effect (also see [132]). These results further implicate the 5-HT_7 receptor. The increase in glucocorticoid receptor expression is mimicked with 5-methoxytryptamine (5-MeOT); an effect blocked with methiothepin as well as H8, an inhibitor of PKA (cyclic nucleotide-dependent protein kinase). Over the course of these studies we found that other serotonergic agonists could partially mimic the 5-HT effect on GR levels, however, this was the first evidence that a more selective serotonergic agonist, 5-CT, could fully mimic the 5-HT effect. Moreover, across all studies, the magnitude of the serotonergic effect on cAMP concentrations is highly correlated ($r = 0.97$) with that on glucocorticoid receptor expression [124]. This observation is consistent with the idea that the effect of 5-HT of glucocorticoid receptor expression in hippocampal neurons is mediated by a 5-HT_7 receptor via activation of cAMP. Interestingly, antidepressant drugs, including some such as amytriptyline that bind with high affinity to the 5-HT_7 receptor, also increase glucocorticoid receptor expression in cultured hippocampal neurons [5,99,148,158]. Antidepressants are known to increase expression of cyclic-nucleotide-dependent response element binding protein (CREB; [208]). Finally, there is evidence for specificity of genomic target. Expression of the closely related mineralocorticoid receptor gene is not affected by postnatal handling or by variations in maternal care [123], nor is there any effect in vitro effect of 5-HT on mineralocorticoid receptor expression in cultured hippocampal neurons [130].

Importantly, the increase in glucocorticoid receptor binding capacity following 5-HT treatment persists following 5-HT removal from the medium; for as long as the cultures can be maintained, there is a sustained increase in glucocorticoid receptor levels as long as 50 days beyond the removal of 5-HT from the medium. Thus, 5-HT can act directly on hippocampal neurons to increase glucocorticoid receptor expression and the effect of 5-HT on glucocorticoid receptor density observed in hippocampal culture cells mimics the long-term effects of early environmental events.

Activation of cAMP pathways is well known to regulate gene transcription through effects on a number of transcription factors, including of course, CREB via an enhanced phosphorylation of CREB e.g. [41]. pCREB regulates gene transcription through pathways that involve the transcriptional cofactor, CREB-binding protein (CBP). Primary hippocampal cell cultures treated with 8-bromo cAMP, 5-CT or 5-HT show a significant increase CBP expression.

The 5-HT$_7$ receptor is positively coupled to adenylyl cyclase [158a]. In vivo, both handling and increased maternal licking/grooming result in an increased level of hippocampal cAMP concentrations and the activation of PKA over the first week of postnatal life [132]. Activation of PKA results in the tissue-specific induction of a number of transcription factors. The day 6 offspring of High LG mothers or pups of the same age exposed to handling show increased hippocampal expression of NGFI-A (aka, zif-268, krox-24, egr-1, zenk, etc; [125]; Weaver et al., submitted). In vitro, 5-HT increases NGFI-A expression in cultured hippocampal neurons and the effect of 5-HT on glucocorticoid receptor expression in hippocampal cultures is completely blocked by concurrent treatment with an oligonucleotide antisense directed at the NGFI-A mRNA [77].

These studies suggest that maternal licking/grooming results in an increased expression of NGFI-A, which in turn might then regulate glucocorticoid receptor expression. Other rodent models examining environmental regulation of hippocampal glucocorticoid receptor expression also suggest a correspondence between NGFI-A levels and glucocorticoid receptor expression [2,135]. In each case, increased levels of NGFI-A are associated with enhanced glucocorticoid receptor expression. However, the critical site for glucocorticoid receptor regulation remained to be defined.

3.1. Glucocorticoid receptor gene regulation

The promoter region of the glucocorticoid receptor gene seemed a reasonable target for the effects of early experience on glucocorticoid receptor expression. Accordingly, we ([118] and see Fig. 3) identified and characterized several new glucocorticoid receptor mRNAs cloned from rat hippocampus. All encode a common protein, but differ in their 5′-leader sequences presumably as a consequence of alternative splicing of potentially, several different sequences from the 5′ non-coding exon 1 region of the glucocorticoid receptor gene. The alternate exon 1 sequences are unlikely to alter the amino acid sequence of the glucocorticoid receptor protein; there is an in-frame stop codon present immediately 5′ to the translation initiation site in exon 2, common to all the mRNA variants. Four of the 10 alternate exon 1 sequences identified by 5′-RACE, exons 1_1, 1_5, 1_9, and 1_{10}, correspond to exon 1 sequences previously identified in the mouse [33,199]. There is a consensus 5′ splice site immediately downstream of each of these exon 1 sequences. Thus, each alternative exon 1 is spliced onto the first coding exon to create diverse glucocorticoid receptor mRNAs. Most alternative exons are located in a 3-kb CpG island upstream of exon 2 that exhibits substantial promoter activity in transfected cells. Ribonuclease protection assays demonstrate significant levels of six alternative exon 1 sequences in vivo in the rat, with differential expression in the liver, hippocampus and thymus presumably

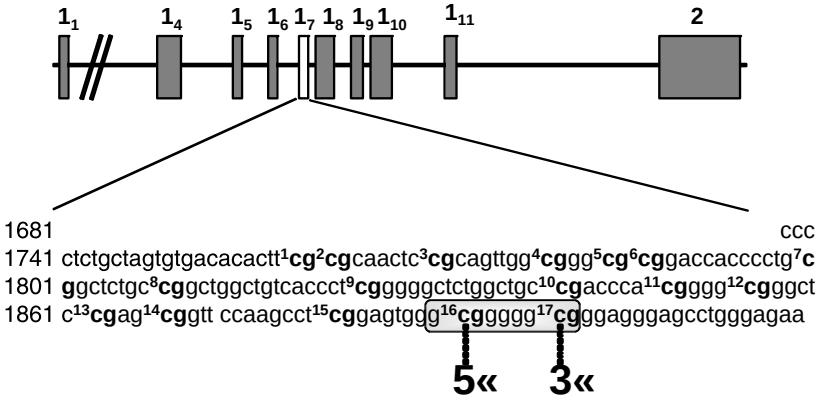

Fig. 3. The 5′ non-coding variable exon 1 region of the hippocampal glucocorticoid receptor gene contains multiple alternate exon 1 sequences [118], four of which correspond to alternative exon 1 sequences previously identified in mouse, exons 1_1, 1_5, 1_9 and 1_{10}. Transfection studies show that the activity of individual constructs fused to a luciferase reporter in different cell types is similar with one notable exception; the exon 1_7 promoter sequence has the highest activity of any single promoter construct [118]. The lower portion provides a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (bold) and the NGFI-A consensus sequence (encircled) with the 5′ and 3′ CpG sites indicated.

reflecting tissue-specific differences in promoter activity. Hippocampal RNA contains significant levels of the exon 1_7-containing glucocorticoid receptor mRNA variants expressed at undetectable levels in liver and thymus.

In transient transfection experiments, a construct encoding the whole CpG island of the glucocorticoid receptor gene, including 8 of the alternate exon 1 sequences, fused to a luciferase reporter gene within exon 2, exhibits substantial promoter activity in neuroblastoma and hepatic cell lines [118]. This activity results from transcripts originating at any point within the CpG island that are spliced from an appropriate donor site onto the splice acceptor site 5′ to exon 2 and represents the sum of the activity of individual promoters on the genomic DNA fragment. Relative activity of these constructs in different cell types is similar with one notable exception, the exon 1_7 promoter sequence. The exon 1_7, fused to luciferase within exon 1_7, has the highest activity of any single promoter construct in B103 and C6 cells, both of which are CNS derived (and see below). The activity of this construct is lower in hepatic cells. In vivo, glucocorticoid receptor mRNA transcripts containing exon 1_7 are present at significant levels in hippocampus, but absent from liver, suggesting that factors present in cells of CNS origin are responsible for transcription initiation at the promoter upstream of exon 1_7 in rat hippocampus.

The results of splice variant analyses suggest that exon 1_7 activity is altered by postnatal handling, which increases glucocorticoid receptor expression in the hippocampus. Handling selectively elevates glucocorticoid receptor mRNA containing exon 1_7 with no effect on exon 1_6 or 1_{10} [118]. Predictably, maternal care also affects the expression of glucocorticoid receptor splice variants: Levels of variants containing the exon 1_7 sequence are significantly increased in the adult offspring of High LG-ABN mothers [Weaver et al., submitted].

3.2. NGFI-A regulation of glucocortioid receptor gene expression

A revealing moment in the cloning studies came with the identification of a core consensus site for NGFI-A (GCGGGGGCG; [36]), within exon 1_7. Thus, increases in NGFI-A induced by maternal licking/grooming could increase transcription from a promoter adjacent to exon 1_7, leading to increased glucocorticoid receptor mRNA. Previous studies revealed that handling increased the binding of both NGFI-A to a promoter sequence for the human glucocorticoid receptor containing consensus sequences for both transcription factors [218]. Since neonatal handling increases maternal licking/grooming, these finding suggest that naturally occurring variations in maternal behavior might regulate glucocorticoid receptor expression in neonatal offspring through a 5-HT-induced increase in NGFI-A expression, and the subsequent binding of NGFI-A to the exon 1_7 promoter. Recent findings support this idea, including studies using chromatin immunoprecipitation (ChIP) assay in which the in vivo formation of protein–DNA complexes are examined using cross-linking with paraformaldehyde perfusion and subsequent precipitation from soluble hippocampal samples using specific antibodies. Protein binding, defined by the specificity of the antibody to specific DNA sequences is then quantified following PCR amplification with targeted primers and Southern blotting. ChIP analysis of hippocampal samples from postnatal day 6 pups reveals dramatically increased NGFI-A binding to the exon 1_7 promoter in the offspring of High compared with Low LG-ABN mothers. These findings confirm that maternal care regulates the binding of NGFI-A to the exon 1_7 promoter sequence in pups.

More recent studies confirm the transactivational effect of NGFI-A at the exon 1_7 sequence using a co-transfection model with HEK cells (intentionally aiming as far from the neural target as possible) with an NGFI-A expression vector and an exon 1_7-luciferase construct. Co-transfection of the NGFI-A vector and the exon1_7-luciferase construct results in a robust increase in luciferase activity, reflecting NGFI-A induced-activation of transcription through the exon 1_7 promoter. Recall that an NGFI-A antisense completely blocks the effects of 5-HT on glucocorticoid receptor expression in hippocampal cell cultures [77].

These findings suggest that NGFI-A might increase glucocorticoid receptor expression in hippocampal neurons, and provide a mechanism for the effect of maternal care over the first week of life. However, while there are striking differences in NGFI-A expression in the offspring of High and Low LG mothers at day 6 of postnatal life, hippocampal NGFI-A expression in adulthood is unaffected by maternal care: There is no difference in hippocampal NGFI-A expression in the adult offspring of High and Low LG-ABN dams. We are thus left with the defining question of early experience studies: How are the effects of early life events, once induced, sustained into adulthood.

4. The epigenome and epigenetic programming of stress responses

DNA is commonly packaged into nucleosomes that involve a close relationship between DNA wrapped around a core of histone proteins [113,210]. The conformation or structure of the histone–DNA configuration regulates gene expression [201]. The relation between DNA and histone is maintained, in part, by electrostatic bonds occurring between the positively-charged histones and the negatively-charged DNA. This 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, histone acetyltransferase (HAT; [180]), catalyze the acetylation of selected amino acids on the protruding histone tails, most commonly histone 3 (H3) or H4. Lysine and arginine residues of the histone tails are common targets for acetylation. Histone acetylation modifies the histone–DNA relation [71]. For example, acetylation of the lysine-9

(K9) residue on H3 neutralizes the positively-charged histone, opening the histone–DNA relationship, and facilitating transcription factor binding to DNA. Thus, H3-K9 acetylation serves as a marker of active gene transcription. Many known transcriptional co-factors, such as the CREB-binding protein (CBP), are HATs [90,133,147,216]. Histone acetylation is dynamic and is regulated by histone deacetylases (HDACs), which serve to block histone acetylation and suppress gene expression [71]. Thus, chromatin structure can be viewed as dynamic and subject to modification through intracellular signals that trigger either HATs or HDACs downstream [10,11,106,202]. The study of histone acetylation greatly advances our understanding of the dynamic and complex regulation of gene expression. Likewise, histone modifications involving methylation, phosphorylation, ribosylation and ubiquitination can all modify chromatin structure and thus gene expression ([210] for a review). In addition to histone modification ATP-dependent chromatin remodeling [214a] occurs through ATPase subunits such as the yeast SWI/SNF complex (with mammalian homologs being the brahma and brahma-related gene-1). However, such histone–DNA modifications are transient modifications occurring in response to specific intracellular signals (e.g., ligand association/dissociation with nuclear receptors; [3a]). This is not the stuff of which stable changes in chromatin structure and gene expression are made.

4.1. The chemistry of DNA methylation

In addition to chromatin, the DNA itself is chemically modified by the addition of methyl residues at the 5′ position of the cytosine rings in the sequence CG in vertebrates [10,168,222]. What distinguishes DNA methylation in vertebrate genomes is the fact that not all CGs are methylated in any given cell type [170,171]. Different CGs are methylated in different cell types, generating cell type specific patterns of methylation. Thus, the DNA methylation pattern confers upon a genome itself a cell-type identity. Since DNA methylation is part of the chemical structure of the DNA, sustained by high energy carbon–carbon bonds, it remains long after all other proteins and epigenomic marks are degraded [6,194], and thus could serve as basis for sustained, ''programming'' effects on gene expression.

DNA methylation patterns were commonly thought to be established only during development and then maintained faithfully through life by the maintenance DNA methyltransferase [171,172]. The DNA methylation reaction was considered irreversible; the only way methyl residues were thought to be lost was through replication in the absence of DNA methyltransferase (i.e., passive demethylation; [168,222]). This mechanism is clearly not applicable to postmitotic tissue such as neurons. However, recent data and those reviewed here support an alternative model that views the DNA methylation pattern as a dynamic equilibrium of methylation and demethylation reaction [205]. We propose that DNA methylation is a reversible signal, like any other biological signal, and could therefore potentially change in response to environmental and physiological events [167]. The notion that DNA methylation is reversible in postmitotic cells has immense implications for our understanding the potential role of DNA methylation in marking gene expression in the brain in response to variations in environmental conditions.

The hallmark of DNA methylation patterns is the correlation between the DNA methylation pattern and chromatin structure. Active chromatin is usually associated with unmethylated DNA, while inactive chromatin is associated with methylated DNA [11,89,92,170]. This link between DNA methylation and chromatin structure has important implications for our understanding of the function of DNA methylation as well as the processes responsible for generating, maintaining and altering DNA methylation patterns under physiological and pathological conditions. It was originally believed that DNA methylation precedes and is dominant over chromatin structure (e.g. [72]). Methylation was thought to be generated independent of chromatin structure. Methylated DNA attracts methylated DNA binding proteins, which recruit repressor complexes containing histone deacetylases, resulting in inactive chromatin [88,139]. The model positioning DNA methylation as driving chromatin inactivation is widespread and profoundly influences our understanding of how altered DNA methylation is involved in cancer. Nevertheless, there are currently data suggesting that chromatin structure can determine DNA methylation, in both directions triggering either de novo DNA methylation or demethylation [25,26,46,47]. These findings revise the classic model of a DNA methylation pattern that is predetermined during development and then maintained through life. The revision suggests a more dynamic view of the DNA methylation pattern as an interface between the dynamic environment and the static genome. Although DNA methylation is an extremely stable signal, it can be altered later in life when there is a sufficiently stable and consistent signal to activate the chromatin. Thus, transient changes in chromatin structure, which commonly accompany altered rates of gene expression, are not accompanied by changes in DNA methylation. Indeed, the DNA methylation pattern is proposed to guard the epigenome from random noise and protect it from drifting [205,206]. The critical feature of the revised model is the potential reversibility of DNA methylation patterns in postmitotic cells through an active process of demethylation/re-methylation in response to a sufficiently potent and persistent signal. This view suggests that the relation between chromatin state and DNA methylation could form a molecular link through which environmental signals might alter DNA methylation in specific genes in postmitotic cells, including neurons.

Environmental signals trigger cellular signaling pathways, the downstream consequence of which is the activation of trans-acting factors. These trans-acting factors recruit can HATs to the target gene resulting in histone acetylation, chromatin opening and, potentially, increased

accessibility to DNA demethylases. Cytosine methylation is an extremely stable chemical bond on DNA. Indeed, for methylation signals to serve as stable marks and potentially as a mechanism for the 'programming' effects of early experience, such marks should not be responsive to chromatin noise or short-term signals. However, the mechanism proposed here also allows for a reversal of the methylation mark later in life by a sufficiently intense and sustained change in chromatin structure [121,205,206].

4.2. How does DNA methylation silence gene expression?

DNA methylation marks genes for silencing by a number of mechanisms. The first mechanism is indirect and links DNA methylation to inactive chromatin structure. A region of methylated DNA juxtaposed to regulatory regions of genes attracts different members of a family of methylated DNA binding proteins. The better-studied member of this class is methylated cytosine binding protein 2 (MeCP2), which recruits HDACs [88,139] and histone methyltransferases [66] to methylated genes [169,11]. This results in a modification of chromatin around the gene precipitating an inactive chromatin structure. A different mechanism involves direct interference of a specific methylated CG residing within a cis recognition sequence for a transcription factor with the interaction of a transcription factors, such as the inhibition of binding of c-Myc to its recognition element when it is methylated [165]. Essentially the methylated cytosine serves as a mutation of the recognition element. Methylation blocks transcription by preventing the interaction of a critical transcription factor with the transcription machinery. Note that blocking the interaction of a transcription factor by methylation would not necessarily cause a change in chromatin structure. In such cases a site-specific methylation blocks gene expression even if the chromatin is in the open configuration. A change in chromatin structure would require the recruitment of HDACs, such as described above. A third mechanism involves a combination of binding of a methylated DNA binding protein and inhibition of activity of a transcription factor [95]. While the first mechanism is dependent on the general density of methyl cytosines within the region associated with a gene rather than methylation of a specific CG, the second mechanism requires a discrete methylation event.

4.3. Maternal care and DNA methylation

Alterations in DNA methylation might provide one mechanism for environmental programming effects occurring in early development. Glucocorticoid receptor gene expression is increased throughout the hippocampus in the adult offspring of High compared with Low LG-ABN mothers ([110,217], Weaver et al., submitted). The exon 1_7 glucocorticoid receptor promoter sequence appears to be significantly more active in the adult offspring of High compared with Low LG-ABN mothers and was therefore the focus of initial studies of possible maternal effects on DNA methylation. To test the hypothesis that maternal care alters the DNA methylation mark of the glucocorticoid receptor promoter, Weaver et al. [217] examined the level of methylation across the entire exon 1_7 glucocorticoid receptor promoter sequence in the hippocampus using sodium bisulfite mapping in the adult offspring of High and Low LG-ABN mothers. Sodium bisulfite (NaBis) treatment of DNA samples converts non-methylated cytosines to uracils, which are then detected as thymidine on subsequent sequencing gels [35,65]. Methylated cytosines are unaffected by NaBis and the differences in methylation status are thus apparent and easily quantifiable on sequencing gels. We found significantly greater methylation of the exon 1_7 glucocorticoid receptor promoter sequence in the offspring of the Low LG-ABN mothers. These findings are consistent with the hypothesis that maternal effects alter DNA methylation patterns in the offspring.

To determine whether DNA methylation of specific target sites on the glucocorticoid receptor promoter change in response to maternal care, we mapped the differences in methylation using NaBis mapping, focusing on a region around the NGFI-A consensus sequence within the exon 1_7 promoter. The results reveal significant differences in the methylation of specific regions of the exon 1_7 glucocorticoid receptor promoter sequence. Notably, the cytosine within the 5′ CpG dinucleotide of the NGFI-A consensus sequence is always methylated in the offspring Low LG-ABN mothers, and rarely methylated in the offspring of High LG-ABN dams. This is consistent with site-specific DNA methylation silencing of the glucocorticoid receptor promoter.

To directly examine a causal relation between maternal behavior and DNA methylation changes within the exon 1_7 glucocorticoid receptor promoter, we [217] performed an adoption study in which the biological offspring of High or Low LG-ABN mothers were cross-fostered to either High or Low dams within 12 h of birth (as described above; [11,58]). These studies could rule out either a purely genetic or a prenatal basis for the variation in DNA methylation in the offspring of High-LG-ABN versus Low-LG-ABN. Cross-fostering the biological offspring of High or Low LG-ABN mothers produced a pattern of exon 1_7 glucocorticoid receptor promoter methylation associated with the rearing mother. The cross-fostering procedure reverses the difference in methylation at specific cytosines. The cytosine within the 5′ CpG dinucleotide of the NGFI-A consensus sequence is hypomethylated following cross-fostering of offspring of Low LG-ABN to High LG-ABN dams, with no effect at the cytosine within the 3′ CpG dinucleotide. Thus, the pattern of methylation of the cytosine within the 5′ CpG dinucleotide of the NGFI-A consensus sequence within the exon 1_7 glucocorticoid receptor promoter of the biological offspring of Low LG-ABN mothers cross-fostered to High LG-ABN dams is indistinguishable from that of the biological offspring of High LG-ABN mothers. The reverse is true for the offspring of High

LG-ABN mothers fostered to Low LG-ABN dams. Interestingly, cross-fostering does not have the same effect on the CpG methylation status on each individual dinucleotide in the exon 1_7 sequence. For example, the CpG dinucleotide of the AP-1 consensus sequence within the exon 1_7 glucocorticoid receptor promoter of the biological offspring of High LG-ABN mothers cross-fostered to Low LG-ABN dams remains hypomethylated. Whereas, the CpG dinucleotide of the AP-1 consensus sequence within the exon 1_7 glucocorticoid receptor promoter of the biological offspring of Low LG-ABN mothers cross-fostered to High LG-ABN dams remains hypermethylated. The molecular basis for such selectivity is unknown, although the specificity suggests that different mechanisms may mediate the effects of maternal care on cytosine methylation across the exon 1_7 sequence, and perhaps an active targeting process is involved in site-specific methylation events (see below). In summary, these findings suggest that variations in maternal care alter the methylation status within specific sites of the exon 1_7 promoter of the glucocorticoid receptor gene and represent the first demonstration of a DNA methylation pattern established through a behavioral mode of programming. Parental imprinting [182], a well-established paradigm of inheritance of an epigenomic mark, requires germ-line transmission [106,172].

4.4. Site-specific methylation of the 5′ CpG dinucleotide of the NGFI-A response element blocks transcription factor binding

The obvious question concerns the functional importance of such differences in methylation. As discussed above, DNA methylation affects gene expression either by attracting methylated DNA binding proteins to a densely methylated region of a gene or by site-specific interference with the binding of a transcription factor to its recognition element [11,169]. The site-specific differences in methylation of the cytosine within the 5′ CpG dinucleotide of the NGFI-A response element is consistent with the hypothesis that methylation at this site interferes with the binding of NGFI-A protein to its binding site. To address this question, Weaver et al. [219] determined the in vitro binding of increasing concentrations of purified recombinant NGFI-A protein [128] to its response element under different states of methylation using the electrophilic mobility shift assay (EMSA) technique with four ^{32}P-labeled synthetic oligonucleotide sequences bearing the NGFI-A binding site that was either: (a) non-methylated, (b) methylated in the 3′ CpG site, (c) methylated in the 5′ CpG site, (d) methylated in both sites, or (e) mutated at the two CpGs with an adenosine replacing the cytosines. NGFI-A formed a protein–DNA complex with the non-methylated oligonucleotide, while the protein is unable to form a complex with either a fully methylated sequence or a sequence methylated at the 5′ CpG site. NGFI-A binding to its response element was only slightly reduced with the sequence methylated at the 3′ CpG site. The effect of selective cytosine methylation on NGFI-A binding was further confirmed by competition experiments. NGFI-A recombinant protein was incubated with a labeled, non-methylated oligonucleotide in the presence of an increasing concentrations of non-labeled oligonucleotides containing the NGFI-A consensus sequence that were either 3′ CpG methylated, 5′ CpG methylated, methylated at both sites, or mutated at the two CpGs with an adenosine replacing the cytosines. The non-methylated oligonucleotide completely eliminates the formation labeled oligonucleotide protein–DNA complex, while the mutated oligonucleotide is unable to compete away the labeled oligonucleotide protein–DNA complex. Neither the oligonucleotide methylated in both the 3′ and 5′ CpGs nor the 5′ CpG methylated oligonucleotide were able to compete. Importantly, the 3′ CpG methylated oligonucleotide, which mimics the sequence observed in the offspring of High LG-ABN mothers, exhibited substantial competition suggesting that binding activity is retained despite the methylation at this site. The results indicate that while methylation of the cytosine within the 5′ CpG dinucleotide reduces NGFI-A protein binding to the same extent as methylation in both CpG sites, methylation of the cytosine within the 3′ CpG dinucleotide only partially reduces NGFI-A protein binding. These data support the hypothesis that methylation of the cytosine within the 5′ CpG dinucleotide in the NGFI-A response element of the exon 1_7 glucocorticoid receptor promoter region in the offspring of Low LG-ABN mothers inhibits NGFI-A protein binding.

This is an important finding for our understanding of the processes by which maternal care programs hippocampal glucocorticoid receptor expression and thus HPA responses to stress. While there are substantial differences in NGFI-A expression between the offspring of High and Low LG-ABN mothers in early postnatal life, no such differences are apparent in adulthood. The obvious hypothesis is that the cytosine methylation of the binding site for NGFI-A interferes with NGFI-A binding to the glucocorticoid receptor exon 1_7 promoter by rendering the sequence a ''low affinity'' site. The prediction is that the lower cytosine methylation in the adult offspring of High compared to Low LG-ABN mothers would result in greater NGFI-A binding to the exon 1_7 promoter. This prediction was confirmed using a chromatin-immunoprecipitation (ChIP) assay examining in vivo formation of protein–DNA complexes in hippocampal tissue from adult animals [217]. Animals were perfused with paraformaldehyde to fix protein–DNA complexes at the time of sacrifice. NGFI-A bound DNA complexes were then immunoprecipitated using a selective antibody. The protein–DNA complexes were uncross-linked, and the precipitated genomic DNA subjected to PCR amplification with primers specific for the exon 1_7 glucocorticoid receptor promoter sequence. The results indicate a 3-fold greater binding of NGFI-A protein to the hippocampal exon 1_7 glucocorticoid receptor promoter in the adult offspring of High compared with Low LG-ABN mothers. Studies using the same tissue samples

and an antibody against the acetylated form of H3 reveal dramatically increased acetylated H3 association with the exon 1_7 glucocorticoid receptor promoter in the offspring of the High LG-ABN mothers. As described above, histone acetylation is associated with active states of gene expression. These findings are therefore consistent with the idea of increased NGFI-A binding to the exon 1_7 promoter and increased transcriptional activation.

Studies using a transient co-transfection assay in human HEK 293 cells confirm that DNA methylation inhibits the ability of NGFI-A to activate the exon1_7 promoter in isolation from other potential differences between adult offspring of High-and Low LG-ABN dams. These cells are not of hippocampal origin and thus allow us to measure the transcriptional consequences of interaction of NGFIA with either a methylated or non-methylated version of the glucocorticoid receptor exon 1_7 promoter per se. While transfection of HEK cells containing an exon1_7-luciferase reporter construct with an NGFI-A expression vector significantly increases luciferase activity, this effect is dramatically reduced if the CpG dinucleotides within the exon1_7 sequence are methylated. Moreover, the effect of NGFI-A on transcription through an exon1_7-luciferase reporter construct was almost completely abolished with a point mutation at the 5′ cytosine (a cytosine to adenosine mutation). Taken together these findings suggest that an "epimutation" at a single cytosine within the NGFI-A consensus sequence alters the binding of NGFI-A and might therefore explain the sustained effect of maternal care on hippocampal glucocorticoid receptor expression and HPA responses to stress.

4.5. How does maternal care alter cytosine methylation?

Maternal behavior could either inhibit de novo methylation or stimulate demethylation. Somewhat surprisingly, a rather simple developmental study of the methylation pattern of glucocorticoid receptor exon 1_7 promoter from E20 to day 90 appears to have resolved this issue. High and Low LG-ABN mothers differ in the frequency of pup licking/grooming and arched-back nursing only during the first week of life. Importantly, this period corresponds to the appearance of the difference in DNA methylation in the offspring in studies using NaBis mapping to precisely define the methylation status of the cytosines within the exon 1_7 glucocorticoid receptor promoter over multiple developmental time points. This analysis demonstrates that just before birth, on embryonic day 20, the entire exon 1_7 region is unmethylated in both groups. Strikingly, one day following birth (postnatal day 1) the exon 1_7 glucocorticoid receptor promoter is de novo methylated in both groups. The 5′ and 3′ CpG sites of the exon 1_7 glucocorticoid receptor NGFI-A response element in the offspring of both High and Low LG-ABN mothers, which exhibit differential methylation later in life, are de novo methylated to the same extent. These data show that both the basal state of methylation and the first wave of de novo methylation after birth occur similarly in both groups. Whereas it is generally accepted that DNA methylation patterns are formed prenatally and that de novo methylation occurs early in development, there is at least one documented example of postnatal de novo methylation of the HoxA5 and HoxB5 genes [80]. Recent studies described below reveal an effect of variations in maternal behavior on estrogen receptor α (ERα) expression in the medial preoptic area of the female offspring in the rat [32]. As described above for the glucocorticoid receptor gene, cross-fostering studies suggest a direct effect of maternal care on ERα expression that emerges over the first week following birth. Since similar analyses are not documented for other genes, it is unknown yet whether changes in methylation are common around birth or whether they are unique to these genomic targets. An important feature of these findings is that of the complete absence of cytosine methylation of the exon 1_7 glucocorticoid receptor promoter on embryonic day 20. Since the majority of the pyramidal cells of Ammon's Horn are born between embryonic day 16 and 20, it seems unlikely that methylation patterns, at least on the exon 1_7 promoter of the glucocorticoid receptor, are generated at the time of DNA replication and cell division, as would normally be the case with imprinted genes (and see below).

The differences in the status of methylation of the exon 1_7 glucocorticoid receptor develop between the two groups emerges between postnatal day 1 and 6, which is precisely the period when differences in the maternal behavior of High and Low LG-ABN dams are apparent. There are no differences in maternal licking/grooming between High and Low LG-ABN mothers beyond day 8 [19,28]. By postnatal day 6, the 5′ CpG dinucleotide of the NGFI-A response element is demethylated in the High, but not in the Low LG-ABN group. These findings are consistent with data from the cross-fostering experiment, which illustrates that the differences between the two groups developed following birth in response to variations in maternal behavior. The group difference in CpG dinucleotide methylation then remains consistent through to adulthood. Our findings suggest that the group difference in DNA methylation occurs as a function of a maternal behavior over the first week of life. The results of earlier studies indicated that the first week of postnatal life is indeed a 'critical period' for the effects of early experience on hippocampal glucocorticoid receptor expression [120] and the development of individual differences in stress responses [105].

4.6. Reversal of the maternal effect on glucocorticoid receptor expression and HPA responses to stress

These findings suggest that maternal behavior produces an active demethylation at selected and presumably targeted sites. The resulting demethylation of the 5′ CpG dinucleotide within the NGFI-A response element of the exon 1_7 promoter enhances NGFI-A binding, increasing glucocorticoid receptor gene transcription and altering HPA responses to stress.

These data beg the question of how increased maternal licking/grooming and arched-back nursing might cause demethylation of the glucocorticoid receptor exon 1_7 promoter. As discussed above a testable working hypothesis is that High LG-ABN leads to activation of NGFI-A as a downstream effector of activation of a 5-HT signaling through increase camp and PKA. Increased NGFI-A increases the frequency of occupancy of the otherwise methylated GR exon 1_7 promoter. Although DNA methylation of the NGFI-A site in Day 1 pups reduces the affinity of the NGFI-A site, the increased levels of NGFI-A in the offspring of the High LG-ABN mothers results in NGFI-A binding to the glucocorticoid receptor exon 1_7 promoter (i.e., increased availability of the 'ligand' results in binding to the 'low affinity' site). NGFI-A interaction with the GR exon 1_7 promoter is associated with the recruitment of HATs to the promoter, which leads to increased acetylation and increased accessibility of the GR exon 1_7 promoter to demethylase resulting in DNA demethylation. As discussed above, increased acetylation of a promoter results in replication independent, active demethylation [25,26]. Once the DNA is demethylated, the NGFI-A binding site is converted into a high affinity state and presumably activated by the normal NGFI-A levels present in the adult hippocampus.

This hypothesis predicts that pharmacological activation of chromatin using HDAC inhibitors should also result in activation of GR exon 1_7 promoter demethylation as long as the demethylation machinery is present in adult neurons. It is well established that the enzymes necessary for various histone modifications are present in adult neurons. However, the question is whether the methylation/demethylation machinery is present only in early life, and thus reversibility is unique to this developmental period, or whether methylation/demethylation machinery is present throughout life in postmitotic cells and it is therefore possible to reverse epigenetic marks later in life in response to signals sufficient to activate chromatin structure or by a pharmacological activation of chromatin structure.

Our hypothesis is that the DNA methylation pattern is a steady state of DNA methylation and demethylation, the direction of which is determined by the state of chromatin structure [121,205,206]. This hypothesis predicts that both DNMTs and demethylases are present in adult neurons and that alteration of the chromatin state by either persistent physiological or pharmacological signals should change the state of methylation of a gene in postmitotic tissue such as adult hippocampal neurons. We previously established that pharmacological activation of chromatin structure by HDAC inhibitors could trigger replication independent active demethylation of DNA [25,26,46,47] and proposed that the demethylation of the GR exon 1_7 promoter is driven by histone acetylation and could be activated in adult neurons. This idea leads to an obvious prediction: HDAC inhibition should reverse the effects of cytosine methylation on NGFI-A binding to the exon 1_7 promoter, GR expression and HPA responses to stress. Weaver et al. [217] tested this idea with central infusion of adult offspring of high or low LG-ABN mothers with the HDAC inhibitor, trichostatin A (TSA), for 4 consecutive days. As expected, ChIP assays reveal that TSA infusion significantly increases the level of acetylated H3 at the exon 1_7 site (i.e., HDAC inhibition increased histone acetylation) in the offspring of Low LG-ABN mothers to levels comparable to those observed in the offspring of High LG-ABN mothers. The increased histone acetylation is associated with enhanced NGFI-A binding to the exon 1_7 promoter sequence and completely eliminates the effect of maternal care. As expected, enhanced NGFI-A binding to the exon 1_7 promoter is associated with increased hippocampal glucocorticoid receptor expression. Hippocampal glucocorticoid receptor expression in the TSA-treated adult offspring of low LG-ABN mothers is indistinguishable from that of the High LG-ABN groups. Most important, TSA infusion also eliminates the effect of maternal care on HPA responses to stress. During and following exposure to acute stress, plasma corticosterone levels in TSA-treated offspring of Low LG-ABN mothers are indistinguishable from those of TSA- or vehicle-treated High LG-ABN mothers. Since under normal circumstances there is considerable H3 acetylation and NGFI-A binding at the exon 1_7 sequence in the adult offspring of High LG-ABN mothers, TSA infusion is without effect in these animals.

If indeed the DNA methylation is in a steady state balance of DNA methylation and demethylation reactions, then it should also be possible to reverse DNA methylation in the other direction and cause remethylation of the glucocorticoid receptor exon 1_7 promoter in the adult offspring of the High LG-ABN mothers. Previous in vitro studies show that increasing the levels of the methyl donor for DNA methylation inhibits replication-independent demethylation presumably by tilting the balance of the DNA methylation/demethylation reaction towards methylation [46,47]. We [220] therefore centrally infused the adult offspring with the essential amino acid L-methionine, a precursor to *S*-adenosyl-methionine (AdoMet) that serves as the donor of methyl-groups for DNA methylation. Methionine infusion reverses the effect of maternal behavior on DNA methylation at the 5′ CpG site of the NGFI-A consensus sequence, NGFI-A binding to the exon 1_7 promoter, GR expression and (HPA). These findings further suggest a causal relation among epigenomic state, GR expression and stress responses in the adult offspring. These results demonstrate that in spite of the inherent stability of the epigenomic marks established early in life through behavioral programming, they are potentially reversible in the adult brain.

An important issue concerns the specificity of the TSA or L-methionine infusion effects. HDAC inhibition or methionine administration with the upregulation of SAM might be thought to increase expression of a wide range of genes within the hippocampus. Yet a microarray study of the hippocampal transcriptome suggests otherwise [220]. In these studies, the vehicle and TSA-treated adult

offspring of Low LG-ABN mothers, and the vehicle and methionine-treated offspring of High LG-ABN dams were sacrificed and Affymetrix microarrays were employed to monitor changes in hippocampal expression of 31,099 unique mRNA transcripts. The three different treatment groups were compared to their respective control groups: (i) vehicle-treated offspring of High LG-ABN mothers vs. vehicle-treated offspring of Low LG-ABN dams, (ii) TSA-treated offspring of Low LG-ABN mothers vs. vehicle-treated offspring of Low LG-ABN dams, (iii) methionine-treated offspring of High LG-ABN mothers vs. vehicle-treated offspring of High LG-ABN dams. The results show that expression of less than 3% of the detected transcripts is altered (i.e., $\geqslant$1.5-fold) through either manipulation. These findings suggest that alterations of cytosine methylation in the adult brain through even rather global procedures are surprisingly specific. Neither TSA nor methionine demethylate or methylate DNA. TSA affects chromatin structure and methionine alters SAM levels. These agents are associated with alterations in DNA methylation only if the necessary methylation/demethylation machinery is present on the gene. Thus the specificity of the effects of these global agents is determined by the occupancy of distinct DNA sites by DNMTs and demethylases. It is tempting to speculate that genes that serve critical regulatory functions, such as the glucocorticoid receptor, are persistently associated with the DNA methylation machinery are thus hypersensitive to global signals such as methionine levels and persistent alterations in histone acetylation. It is becoming increasingly clear that chromatin and DNA methylation enzymes are targeted to specific genes in a regulated process. A critical challenge is that of elucidating the mechanisms that target the DNA methylation/demethylation machinery to specific genes, such as the glucocorticoid receptor gene in the hippocampus.

In summary, these findings suggest specific molecular mechanisms linking early maternal behavior and stable changes in behavior later in adulthood. First, these data support the idea that demethylation is driven by activation of chromatin and that HDAC inhibitors produce demethylation even in nondividing cells (i.e., in a replication-independent manner). Second, the findings are consistent with the hypothesis that the demethylation of GR exon 1_7 in offspring of High LG-ABN rats early after birth is driven by increased histone acetylation. Third, these data provide evidence that molecular mechanisms that underlie the effects of early life-experience neural function are potentially reversible in adulthood. This consideration is of obvious social and therapeutic implications. Fourth, these data provide in vivo evidence for our hypothesis that the DNA methylation pattern is dynamic even in postmitotic tissues and that its steady state is maintained by the state of chromatin acetylation [205]. Finally, the data provide a general mechanism for how external signals could change the DNA methylation pattern and thus the chemistry of the genome itself even during adulthood.

5. The transmission of individual differences in maternal behavior

The variations in maternal behavior influence the development of both behavioral and HPA responses to stress in the offspring. These individual differences in maternal behavior are also transmitted to the female offspring. Thus, the female offspring of High LG-ABN mothers are, as adults, High LG-ABN dams; likewise, the lactating adult offspring of Low LG-ABN mothers are Low LG-ABN dams (among rats, at least, females do become their mothers). Cross-fostering studies [58] reveal evidence for a direct effect of maternal care on the development of individual differences in pup LG and ABN among adult females. Thus, as lactating adults, female pups fostered from Low LG-ABN dams to High LG-ABN mothers, exhibit levels of pup LG that are comparable to the normal offspring of High LG-ABN mothers (and vice versa).

The individual differences in maternal behavior appear to be associated with variations in estrogen–oxytocin interactions at the level of the medial preoptic area (MPOA) and downstream effects on mesolimbic dopamine system. Differences in estrogen receptor alpha (ERα) appear critical, and there is evidence that the transmission of individual differences in maternal behavior across generations may be mediated by differential cytosine methylation of a stat5 consensus sequence with the estrogen 1B promoter.

Hormonal changes in the final trimester of pregnancy, including elevations in the levels of estradiol and a marked decline in those of progesterone, form the basis for the rapid, postpartum onset of maternal behavior in the rat [14,55,136,178]. Many of the relevant hormonal effects for maternal behavior (e.g., prolactin, oxytocin) require estrogen [14] effects at the level of the MPOA [52,143,156]. These estrogen effects involve the induction of oxytocin receptor binding [156] through ERα [224]. There occurs a dramatic increase in oxytocin receptor binding in the MPOA on Days 19–21 of pregnancy [156], a period that shortly follows the rise in estrogen levels in late gestation [14]. ICV administration of oxytocin rapidly stimulates maternal behavior in virgin rats [51,154]. The effect of oxytocin is abolished by ovariectomy and re-instated with estrogen treatment. Moreover, treatment with oxytocin-antisera or receptor antagonists blocks the effect of estrogen on maternal behavior [143,155]. Likewise, infusion of an oxytocin receptor antagonist directly into the MPOA reduces maternal behavior [154]. In the pup sensitization paradigm [15], non-maternal virgin females that are continuously housed with pups ultimately exhibit maternal behavior; oxytocin receptor levels in the MPOA predict the latency for the onset of maternal behavior [27].

Lactating High LG-ABN mothers show increased oxytocin receptor binding in the MPOA suggesting greater sensitivity to oxytocin and thus an enhanced feed-forward effect on oxytocin synthesis in the MPOA [86,141]. Indeed, there is an increased number of oxytocin neurons in the MPOA of High compared with Low LG-ABN mothers

[191]. Differences in oxytocin receptor binding in the MPOA are estrogen dependent [27,57]. In the absence of estrogen or in diestrus virgin females there are no differences in oxytocin receptor levels [27,29,57]. In ovariectomized animals, estrogen replacement at levels that mimic those of late pregnancy [14] induces a dose-related increase in oxytocin receptor levels in the MPOA in the offspring of High LG-ABN mothers, but is without effect on the offspring of the Low LG-ABN dams [29]. The same group differences in estrogen sensitivity are apparent in estrogen-induced cFOS expression in the MPOA [29]. Such variations in estrogen sensitivity are likely mediated by tissue-specific differences in estrogen receptor expression in the MPOA. Lactating High LG-ABN mothers show increased ERα expression in the MPOA compared with lactating Low LG-ABN dams; such differences are apparent at the level of both mRNA and protein, with no differences in the expression of ERβ [29]. The estrogen-induced difference in oxytocin receptor binding appears critical; ICV infusion of an oxytocin receptor antagonist on Day 3 postpartum completely eliminates the group difference in pup licking/grooming [27], with no effect on the amount of time spent in contact with pups. It remains to be defined whether the MPOA is the critical site for this effect. Finally, the same differences in ERα expression in the MPOA are apparent in the virgin female offspring of High and Low LG-ABN mothers and are completely reversed with cross fostering; reflecting a direct effect of maternal care on ERα expression. These latter findings suggest a maternal effect on ERα expression in the MPOA that might mediate the transmission of individual differences in maternal behavior from mother to female offspring.

Oxytocin neurons in the MPOA project to the VTA [144,145] and there is behavioral evidence for an effect of oxytocin on dopamine release from the VTA [183]. Double labeling for oxytocin immunoreactivity and tract tracing following flurogold injections directly into the VTA reveals a greatly increased number of oxytocin-neurons with projections (i.e., double labeled cells; [191]) to the VTA in lactating High LG-ABN females. Maternal responsivity in the rat is associated with the activation of nucleus accumbens (nAcc) neurons [56,111] and dopamine receptor antagonists infused into the nAcc significantly reduce the frequency of pup licking/grooming in lactating rats [91]. Lactating female rats bar press vigorously to gain access to pups and responding is eliminated with lesions of the MPOA [100]. High LG-ABN mothers show an increased frequency of pup licking/grooming bouts and such bouts are substantially longer in High LG-ABN mothers [30]. Pup licking/grooming is preceded by an increase in the dopamine signal in the nAcc and remains elevated throughout the licking/grooming bout. The dopamine signal in the nAcc is significantly greater in High LG-ABN dams and the magnitude of the dopamine signal in the nAcc is highly correlated with the duration of the licking/grooming bout ($r = +0.80$; 30). A dopamine transporter blocker eliminates the group differences in the nAcc dopamine signal and pup licking/grooming [30]. Interestingly, there are no differences in oxytocin receptor binding between High and Low LG-ABN females in either the VTA or the nAcc, suggesting that the relevant oxytocin signal emanates from outside the mesolimbic dopamine system; we propose the origin lies in the MPOA. These findings suggest that the differential activation of the VTA projection to the nAcc through, in part, an estrogen-dependent oxytocin signal mediates the differences in pup licking/grooming between High and Low LG-ABN mothers.

Recent studies [32] explored the possibility that variations in maternal care might alter ERα expression in offspring through differential methylation of the relevant promoter sequence. The human (h) ER gene is transcribed from two different promoters, the proximal A and distal B promoter, which are separated by a 2 kb intron. Sequencing analysis of the 5′ flanking untranslated exon (exon 1b) region of the rat ER gene [64] shows that transcription occurs from a promoter >70% homologous to human ER B promoter, with no evidence for a functional promoter A in the rat ER gene. Importantly, the rat ER mRNA transcribed from promoter B is the only ER mRNA detectable in neuronal tissue [185]. These findings suggest that the exon 1b region contains the elements necessary for transcriptional regulation of constitutive expression of the rat ER gene in the brain. NaBis mapping shows decreased cytosine methylation across the entire exon 1B promoter in the offspring of High compared with Low LG-ABN mothers. Of particular interest are the differences within the stat5 binding site of the promoter. Prolactin acts through the JAK2–Stat5 pathway to enhance ERα expression in a variety of tissues [61,62] although to the best of our knowledge this effect has never been examined in the MPOA.

Cytosine methylation is associated with decreased transcription factor binding to DNA consensus sequences. The differences in cytosine methylation of the stat5b binding site would therefore suggest differences in stat5b binding in adult offspring as a function of maternal care. The ERα promoter B contains a stat5 consensus sequence, the activation of which increases ERα expression [94]. Among adult animals, there is increased stat5b binding to the ERα promoter B in the MPOA of offspring of High compared to Low LG-ABN mothers. This difference occurs in response to comparable, basal levels of prolactin in non-lactating, adult females. Indeed, we found no differences in circulating prolactin levels or in the expression of the long or short variant of the prolactin receptor in the MPOA of High and Low LG-ABN females. The obvious working hypothesis is that enhanced maternal licking/grooming in early life leads to increased stat5b expression in the MPOA that targets 'demethylation' of the stat5b binding site, permitting greater stat5b binding in response to subsequent activation of the prolactin–JAK/stat pathway and thus increased ERα expression. These findings suggest that individual differences in maternal behavior are transmitted across generations through maternal effects on the methylation status of the Stat5 site in the ERα 1B promoter in the MPOA.

6. Dissection of the molecular mechanisms linking maternal behavior and active demethylation of GR exon 1_7 promoter in the hippocampus

The data discussed above suggests that histone acetylation could produce the conditions facilitating active demethylation of the GR exon 1_7 promoter. Yet several questions remain unanswered. How, for example, is histone acetylation targeted to the exon 1_7 promoter as a consequence of maternal behavior? We proposed above that maternal behavior stimulates 5-HT, which in turn stimulates NGFI-A, and that NGFI-A then targets HATS and eventually demethylases to the glucocorticoid receptor exon 1_7 promoter Studies with hippocampal primary neuronal cell cultures as well as nonneuronal cell lines have started to address this question. The two systems have different strengths and could be used to test different components of the model. The first set of studies examined the hypothesis that 5-HT acts through cAMP to produce hypomethylation. Hippocampal cell cultures treated with either 5-HT or 8-bromo-cAMP, a stable cAMP analog, show increased glucocorticoid receptor expression following 4 days of treatment [77,130,132]. Treatment of hippocampal cells in culture with 5-HT also results in the hypomethylation of the 5′ CpG dinucleotide of the NGFI-A consensus sequence within the exon 1_7 promoter of the glucocorticoid receptor gene, with no effect at the 3′ site [Weaver et al., 2005]. Treatment with 8-bromo-cAMP produces an even more pronounced effect on cytosine methylation at the 5′ CpG site. In both studies, cultures maintained under control conditions show complete methylation of both the 5′ and 3′ CpG sites of the NGFI-A consensus sequence. Bromo-deoxyuridine labeling, which marks newly generated cells, reveals little or no cell replication in the cultures at the time of 5-HT treatment. These findings reinforce the idea that the alterations in cytosine methylation occur independently of cell replication and in response to intracellular signals associated with variations in maternal care. The cell culture studies establish that 5-HT signaling induced by maternal care can trigger replication-independent changes in methylation of GR exon 1_7 promoter through an increase in cAMP. Since increased cAMP activates NGFI-A, it seems that ectopic expression of NGFI-A might target the demethylation process to the GR exon 1_7 promoter. However, this would not explain the selective effect at the 5′ cytosine.

Studies with human embryonic kidney (HEK 293) cells further tested the hypothesis that NGFI-A targets demethylation to GR exon 1_7 promoter. Use of the HEK cells permits the isolation of the direct effect of NGFI-A distinct from other neuron-specific events that might confound the interpretation of data from the hippocampal cultures. Comparison of the fate of a transiently transfected methylated GR exon 1_7 promoter-luciferase vector in the presence and absence of NGFI-A permits a better determination of the specific effects of NGFI-A on demethylation. Since the nonintegrated plasmid does not bear an origin of replication and does not replicate in HEK 293 cells, the assay measures the effects of NGFI-A on active replication-independent demethylation. Whereas in vitro the methylated glucocorticoid receptor exon 1_7 promoter-luciferase vector remains methylated in HEK 293 cells, co-expression of NGFI-A results in active demethylation of a significant fraction of the transfected plasmids. A site-direct mutagenesis of the two CpGs included in the NGFI-A recognition element was performed to demonstrate that DNA demethylation requires direct contact between NGFI-A and its recognition element. The preliminary results suggest that these manipulations abolish the ability of NGFI-A to activate and demethylate the glucocorticoid receptor exon 1_7 promoter [Weaver et al., unpublished data]. These experiments provide a molecular mechanism on how demethylation is triggered to specific sequences. The outstanding question is to determine how NGFI-A triggers demethylation upon binding to specific sequences. One possibility is that NGFI-A directly recruits a demethylase to the gene or that as proposed before it recruits a HAT which increases acetylation thus increasing the accessibility to demethylase as proposed before [25,26,46,47]. Interestingly, NGFI-A can actively target methylated DNA binding proteins to genomic targets [23]. NGFI-A might also recruit a HAT such as CBP. Not surprisingly both 5-HT and 8-bromo-cAMP increase CBP expression in hippocampal neurons and, in vivo, there is increased CBP binding to the glucocorticoid receptor exon 1_7 promoter in the neonatal offspring of High LG-ABN mothers. The exon 1_7 promoter does not contain a CREB binding site and the variations in maternal care described here do not seem to affect the expression of either CREB or pCREB. However, there is evidence that the N- and C-terminal domains of CBP directly interact with the transcriptional domain of NGFI-A [195] suggesting that protein–protein interactions might directly recruit CBP and thus HAT activity. Histone acetylation associated with CBP might then increase the accessibility of the exon 1_7 promoter to demethylase. Previous studies show that histone acetylation can trigger replication-independent demethylation of previously methylated promoters in HEK293 cells [25,26].

The issue of active DNA demethylation as a mechanism for gene activation has generally received less emphasis that the well-established relation between DNA methylation and gene silencing. Evidence for the importance of active DNA demethylation is not unique to our studies. A recent paper on altered expression of IL-2 expression by T lymphocytes following activation clearly implicates an active process of demethylation in a normal nontransformed somatic cell. Bruniquel and Schwartz [17] found that a region in a promoter of the IL-2 gene demethylates following activation in the absence of DNA replication and results in a profound increase in the production of IL-2. Taken together these studies have revised interest in active demethylation and, not surprisingly, the hunt for the relevant enzymatic machinery [10a].

6.1. The putative demethylase

The results of the studies with hippocampal cell cultures the HEK 293 cells and the developmental time course study suggest a process of active demethylation. In the developmental studies, the 5′ CpG site is initially methylated to the same extent in the offspring of High and Low LG-ABN mothers. Over the course of the first week following birth the methylation mark is functionally removed from the 5′ site in the offspring of High LG-ABN mothers. Prevailing models of DNA methylation propose that the methylation pattern of newly synthesized DNA is exclusively determined by the methylation pattern of the parental strand, and thus thought to be preserved in somatic cells. This model cannot explain the data described here or recent data demonstrating demethylation in response to changes in chromatin structure, such as those induced by TSA [25]. In response to such findings, Szyf and colleagues [8,167] proposed that DNA methylation is enzymatically reversible and that DNA methylation is dynamic in fully differentiated cells. This idea remains controversial. Active demethylation was nevertheless clearly demonstrated early in embryogenesis and the parental genome undergoes replication independent, active demethylation hours after fertilization, well before the initiation of replication [150]. Demethylation at very early stages in development has been relatively accepted, but the possibility of postnatal demethylation, and especially in fully differentiated somatic cells, has been hotly disputed. However, active replication demethylation was demonstrated in EBV infected B cells [207] and more recently it was repeatedly demonstrated in HEK293 cells [25,26,45,46]. The HEK 293 transient transfection provided direct evidence that active replication-independent demethylation takes place in somatic cells and that it is dependent on chromatin state. Fully in vitro methylated plasmid is transiently transfected into the cells. The plasmid, which does not bear an origin of replication, does not replicate in these cells and that has been validated using a *Dpn*I restriction analysis [25]. *Dpn*I cleaves plasmids only with a preserved bacterial pattern of DNA methylation. Plasmids are raised in bacteria and lose their methylation pattern once they replicate in mammalian cells, which do not express the bacterial methylation enzymes [25]. Upon treatment of the cells with TSA the plasmid undergoes complete demethylation that could only be accomplished by a processive demethylase [25].

Earlier studies from Szyf's laboratory extracted active DNA demethylase activity from a human lung cancer cell line [167] and identified a protein with demethylase activity [8], which was cloned concurrently by Bird's group and named MBD2 [78]. Interestingly, the protein, MBD2, was found by Bird's group and others to also associate with a chromatin remodeling complex containing HDAC, which is involved in silencing of gene expression through the recruitment of a repressor complex. The assignment of a demethylase function to a protein that was independently discovered as a recruiter of repressor complexes triggered the expected controversy in the field and reports that MBD2 failed to produce demethylase activity. However, the observation that MBD2/dMTase expression produces the demethylation of some, but not all promoters in a dose- and time-dependent manner might explain this dual function of MBD2 (e.g. [26,45]). Clearly the contextual factors that determine MBD2 demethylase activity remain to be fully explained. Other data supports a demethylase function for MBD2. Antisense knock down of MBD2 resulted in inhibition of active demethylation induced by valproate [46] and caused hypermethylation and silencing of the prometastatic gene uPA in metastatic breast cancer cells [152]. Another group reported that ectopic expression of MBD2/demethylase in hepatocyte cell line caused demethylation and activation of the hexokinase type 2 gene [67]. Additional support for the demethylase activity of MBD2/demethylase emerges from the finding that expression of MBD2/demethylase is correlated with demethylation within the promoters of C-ERBB-2 and SURVIVIN genes in ovarian cancers [73,74] and hypomethylated CMYC in gastric cancer [53]. In addition, the *Drosophila* homolog of MBD2, dMBD2/3, formed foci that associated with DNA at the cellular blastoderm stage, concurrent with the activation of the embryonic genome, and also associated with the active Y chromosome [115].

To test the hypothesis that MBD2 is associated with maternally-induced demethylation, we performed an in situ hybridization assay with probes for the mRNAs for a number of methylated binding proteins at day 6 postpartum, the time at which the exon 17 promoter is demethylated in the offspring of the High LG-ABN mothers. The analysis reveals increased MBD2/demethylase expression in the hippocampus at this point in time in offspring of High versus Low LG-ABN mothers. In the hippocampal cell culture model both 5-HT and c-AMP significantly increase MBD2/demethylase expression. A ChIP analysis with an anti-MBD2/demethylase antibody demonstrates significantly increased binding of MBD2/dMTase to the exon 1_7 GR promoter in day 6 offspring of High versus Low LG-ABN mothers. Recall there is increased NGFI-A binding to the same sequence in day 6 offspring of High LG-ABN offspring. We then performed a bisulfite mapping of the state of methylation of the exon 1_7 glucocorticoid receptor promoter bound to MBD2 and precipitated in the ChIP assay with anti-MBD2 antibody. If MBD2 is the demethylase involved in this process or if it is part of the demethylase complex, then MBD2-bound exon 1_7 sequences at day 6 should be found in the process of demethylation. Indeed, most of the MBD2-bound DNA was unmethylated or partially unmethylated [Weaver et al., unpublished].

In summary, our findings suggest that shortly after birth there is a wave of de novo methylation that results in the methylation of both CpG sites within the NGFI-A consensus sequence. Such events would impede the binding of NGFI-A to the exon 1_7 promoter. However, in the offspring of the High LG-ABN mothers, NGFI-A expression

is increased to the point where binding occurs despite the "low affinity" status of the binding site. The binding of NGFI-A is associated with histone acetylation and the subsequent availability of the site to demethylase. In support of this idea, the treatment of the adult offspring of the Low LG-ABN mothers with TSA increases H3 acetylation and NGFI-A binding (see above) and results in the demethylation of the 5′CpG site of the NGFI-A consensus sequence [217]. While this model remains speculative at this time, these findings do suggest that modifications to the DNA methylation status in fully differentiated cells are clearly possible and pharmacologically reversible, an idea that holds considerable potential therapeutic implications.

7. Experience-dependent chromatin plasticity? Environmental variability meets epigenomic predictability

The defining question of early experience studies concerns the mechanism by which environmental effects occurring in early development are 'biologically embedded' and thus sustained into adulthood (i.e., so-called 'environmental programming' effects). The offspring of High LG-ABN mothers exhibit increased hippocampal glucocorticoid receptor expression from the exon 1_7 promoter and dampened HPA responses to stress that persists into adulthood. We propose that the differential epigenomic status of the exon 1_7 glucocorticoid receptor promoter in the offspring of High LG-ABN mothers serves as a mechanism for this maternal effect. It is important to note that these findings are restricted to the study of a single promoter of but one gene in one region of the brain, with emerging data on ERα expression in the MPOA of the females. The degree to which such results might generalize to other instances of environmental programming remains to be determined. Moreover, further studies are required to determine how maternal behavior alters the epigenomic status of the exon 1_7 glucocorticoid receptor promoter. The developmental time course study is critical. Recall, the 5′ CpG dinucleotide of the NGFI-A consensus sequence of the exon 1_7 promoter is methylated to the same, elevated level in the newborn offspring of High and Low LG-ABN mothers. It is only over the first week of life that the difference emerges, with the decline in the methylation of the 5′ CpG site in the offspring of High, but not Low LG-ABN mothers. No such demethylation occurs at the neighboring 3′ CpG site. The impressive selectivity suggests a demethylation process that is targeted in some manner. It is critical to define the processes by which such apparently active demethylation might occur. Regardless of these yet unanswered questions, these findings provide evidence that maternal behavior stably alters the epigenome of the offspring, providing a mechanism for the long-term effects of early experience on gene expression in the adult.

Studies of epigenetic modifications offer an opportunity to clearly define the nature of gene–environment interactions during development and how such effects result in the sustained 'environmental programming' of gene expression and function over the life-span. 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 [1,137,179] such that the magnitude of the maternal influence on the development of HPA and behavioral responses to stress in the rat should not be surprising (see [21]). It is important to consider the potential adaptive value of such processes. While enhanced exposure to CRF, catecholamines and glucocorticoids may promote multiple forms of chronic illness, these hormones provide essential adaptive effects for animals living in environments with an increased level of demand. For example, impoverished environments are commonly associated with multiple sources of infection. Under such conditions adrenal glucocorticoids serve as a potent defense against septic shock [138]. Among rats, animals with increased HPA responses to agents such as bacterial endotoxins are at reduced risk for sepsis [144]. Interestingly, adults exposed to a bacterial endotoxin during the first week of life exhibit increased HPA responses to stress as well increased resistance to sepsis upon subsequent exposure to bacterial infection [192,193]. Conversely, postnatal handling, which increases maternal licking/grooming and dampens HPA responses to stress, serves to *increase* vulnerability to endotoxin-induced sepsis [97]. Stress hormone responses also promote detection of potential threat, fear conditioning to stimuli associated with threat and avoidance learning. Under conditions of increased predation, more fearful animals commonly show increased survival (e.g. [149]). Moreover, catecholamines and glucocorticoids mobilize energy reserves through effects of lipolysis, glycolysis and gluconeogenesis. These effects are the hallmark of the shift to catabolism that occurs during periods of stress and are essential for animals exposed to famine. Indeed, the ability to survive sustained periods of nutrient deprivation depends upon the capacity to increase circulating levels of glucocorticoids and catecholamines.

Stress during gestation decreases pup licking/grooming in the rat [31,197]. This pattern of maternal care serves to enhance stress reactivity of the offspring (i.e., the offspring of Low LG-ABN mothers exhibit increased fearfulness and enhanced HPA responses to stress). Under normal lab conditions, Low LG-ABN mothers are more fearful than are High LG-ABN dams [57]. Hence the individual differences in stress reactivity can be transmitted from mother to offspring, and the results of the cross-fostering studies reveal that this process can occur through variations in maternal behavior (i.e., a nongenomic mechanism of inheritance). Such intergenerational transmission of individual differences in stress reactivity via maternal behavior could represent an adaptive approach to development. Since the offspring usually inhabit a niche that is similar to their parents, the transmission of individual differences in traits from parent to offspring could serve to be adaptive with respect to survival. Adversity over the adult life of the parent is thus likely to predict more of the same for the offspring. Indeed,

such maternal effects could result in the transmission of adaptive responses across generations [121,126]. Importantly, stress during gestation decreases pup licking/grooming in High LG-ABN mothers to levels that are indistinguishable from those of Low LG-ABN dams ([31], and also see [197]). Predictably, the offspring of stressed/High LG-ABN mothers show reduced hippocampal GR expression and increased fear behavior [31].

Epigenomic modifications of targeted regulatory sequences in response to even reasonably subtle variations in environmental conditions might serve as a major source of epigenetic variation in gene expression and function and ultimately as a process mediating such maternal effects. We propose that epigenomic changes serve as an intermediate process that imprints dynamic environmental experiences, such as variations in parental care, on the fixed genome resulting in stable alterations in phenotype. Such variations may then serve as a source of individual differences in stress responses and define vulnerability/resistance to chronic illness over the lifespan depending upon the conditions of the prevailing environment. It's a question of fit.

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