Early Environmental Regulation of Hippocampal Glucocorticoid Receptor Gene Expression

Characterization of Intracellular Mediators and Potential Genomic Target Sites

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ABSTRACT: Environmental conditions in early life permanently alter the development of glucocorticoid receptor gene expression in the hippocampus and hypothalamic-pituitary-adrenal responses to acute or chronic stress. In part, these effects can involve an activation of ascending serotonergic pathways and subsequent changes in the expression of transcription factors that might drive glucocorticoid receptor expression in the hippocampus. This paper summarizes the evidence in favor of these pathways as well as recent studies describing regulatory targets within the chromatin structure of the promoter region of the rat hippocampal glucocorticoid receptor gene.

KEYWORDS: maternal care; stress; glucocorticoid receptor; methylation; epigenomic programming

Several years ago Levine, Denenberg, Zarrow, and colleagues[1-6] showed that early experience modified the development of adrenal glucocorticoid responses to a wide range of stressors. These findings clearly demonstrated that even rudimentary adaptive responses to stress could be modified by environmental events. More recent studies[7] revealed these environmental effects produce sustained alterations in glucocorticoid receptor gene expression in the hippocampus and frontal cortex, which mediate glucocorticoid negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis and thus result in stable differences in HPA responses to stress. These studies reflect the plasticity within brain regions that regulate the activity of the HPA axis, and provide a model for understanding the processes that contribute

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to individual differences in neuroendocrine function. The importance of this topic is underscored by the fact that individual differences in adrenal hormone and sympathetic responses to stress appear to be of considerable importance in determining the vulnerability to multiple forms of pathology.[8–11]

ENVIRONMENTAL REGULATION OF HPA AND BEHAVIORAL RESPONSES TO STRESS

Postnatal Handling Studies

Perhaps the strongest evidence for the environmental regulation of the development of HPA responses to stress comes from the postnatal handling research with rodents. Handling involves a brief (i.e., 3–15 min), daily period of separation of the pup from the mother for the first few weeks of life and results in decreased stress reactivity in adulthood.[2–6,12–15] As adults, neonatally handled rats show decreased fearfulness and more modest pituitary ACTH and adrenal corticosterone responses to stress; such effects are apparent in animals tested as late as 26 months of age.[16,17]

The handling effects on the development of HPA responses to stress have important functional consequences. In the rat, glucocorticoid levels often rise with age and are associated with hippocampal degeneration and the emergence of learning and memory deficits.[18–21] Such age-related increases in basal and stress-induced pituitary-adrenal activity are significantly less apparent in the handled animals, and thus these animals show little evidence of hippocampal aging.[16] Likewise, handled animals also show more modest stress-induced suppression of immune function by comparison to non-handled rats.[22]

Such findings may lead to the conclusion that handled animals are hardier or more resistant than non-handled animals. But this misses the point. Handled animals are not better adapted than non-handled animals, they are simply different. The environmental context then serves to determine the adaptive value of increased or decreased stress reactivity. In the examples cited above, it would appear that the handled animals are at some advantage by virtue of a more modest HPA response to stress. But this condition is not universal, Laban and colleagues[23] found that non-handled animals are more resistant to the induction of experimental allergic encephalomyelitis (EAE) than are handled animals. Glucocorticoids are protective against the development of EAE, which can be fatal.[24] Adrenalectomized animals, for example, rarely survive EAE. Hence, the increased HPA responsivity of the non-handled renders an advantage under these circumstances. The cost of such resistance is an increased vulnerability to glucocorticoid-induced illness, but it is not difficult to imagine a scenario whereby such a cost is an acceptable trade-off. In essence, the handling studies represent a robust example of phenotypic plasticity in the expression of defensive responses to threat. One obvious question concerns the nature of the neurobiological mechanisms that mediate such phenotypic variation.

Considering the importance of the corticotropin-releasing hormone (CRH) systems for both behavioral and HPA responses to stress, it is probably not surprising that these systems are critical targets for the handling effect on stress reactivity. Adult animals exposed to postnatal handling show decreased CRH mRNA expression in the paraventricular nucleus of the hypothalamus (PVNh) and the central nucleus of the amygdala,[14,25,26] decreased CRH content in the locus coeruleus,[26] and

decreased CRH receptor levels in the locus coeruleus compared with non-handled rats.[26] The release of CRH and the activation of HPA responses to stress are mediated by stress-induced increases in the release of noradrenaline at the level of the PVNh. Indeed, CRH release from the amygdala activates the release of noradrenaline from the locus coeruleus.[27] Together, these findings suggest that there would be more modest CRH-induced activation of the locus coeruleus during stress in the handled animals. At least two findings are consistent with this idea. By comparison to non-handled rats, acute stress in handled animals produces (1) a smaller stress-induced increase in cFOS immunoreactive neurons in the locus coeruleus[28] and (2) more modest increases in extracellular noradrenaline levels in the PVNh.[29] We propose that postnatal handling can decrease the expression of behavioral responses to stress, in part, by altering the development of the central nucleus of the amygdala–locus coeruleus CRH system.

Postnatal handling affects the development of neural systems that regulate CRH gene expression. Levels of CRH mRNA and protein in PVNh neurons are subject to inhibitory regulation via glucocorticoid negative feedback.[9,30] Handled rats show increased negative feedback sensitivity to glucocorticoids.[13,14] This effect is, in turn, related to the increased glucocorticoid receptor expression in the hippocampus and frontal cortex,[14,31-33] regions known to mediate the inhibitory effects of glucocorticoids over CRH synthesis in PVNh neurons.[19,30,34-36] The alterations in glucocorticoid receptor expression are a critical feature for the effect of the early environment on negative feedback sensitivity and HPA responses to stress; reversing the differences in hippocampal glucocorticoid receptor levels eliminates the differences in HPA responses to stress between handled and non-handled animals.[13]

CRH activity within the amygdala–locus coeruleus pathway is subject to γ-aminobutyric acid-(GABA-)ergic inhibition.[37,38] Interestingly, handled rats also show increased $GABA_A$ and benzodiazepine (BZ) receptor levels in the noradrenergic cell body regions of the locus coeruleus and the nuclei tractus solitarius as well as in the basolateral and central nucleus of the amygdala.[39] These effects are associated with increased expression of the mRNA for the α1 and γ2 subunit of the $GABA_A$ receptor, which together encode for proteins that form the BZ site. These findings suggest that the composition of the $GABA_A$ receptor complex in brain regions that regulate stress reactivity is influenced by early life events. Handling increases α1 and γ2 subunit expression[39,40] and, importantly, this profile is associated with increased GABA binding.[41,42] Interestingly, in humans, individual differences in BZ receptor sensitivity are associated with vulnerability for anxiety disorders.[43]

Together, the effects of handling on glucocorticoid and $GABA_A$/BZ receptor gene expression could serve to dampen CRH synthesis and release and to decrease the effect of CRH at critical target sites, such as the locus coeruleus. We feel that this model provides a reasonable working hypothesis for the mechanisms underlying the handling effect on endocrine and behavioral responses to stress.[7]

WHAT ARE THE CRITICAL FEATURES OF THESE ENVIRONMENTAL MANIPULATIONS?

Some years ago Levine and colleagues[44] suggested that the effects of handling were actually mediated by alterations in mother–infant interactions. Indeed, postna-

tal handling increases the frequency of pup licking/grooming by mothers.[45] We[40,46,47] examined this question by attempting to define naturally occurring variations in maternal behavior over the first eight days following birth through the simple, albeit time-consuming, observation of mother–pup interactions in normally reared animals. There was considerable variation in two forms of maternal behavior: licking/grooming of pups and arched-back nursing.[48] Licking/grooming included both body as well as anogenital licking. Arched-back nursing, also referred to as "crouching," is characterized by a dam nursing her pups with her back conspicuously arched and legs splayed outward. While common, it is not the only posture from which dams nurse. A blanket posture represents a more relaxed version of the arched-back position, where the mother is almost lying on the suckling pups. This position provides substantially less opportunity for movements such as nipple switching. Dams also nurse from their sides and often will move from one posture to another over the course of a nursing bout. Interestingly, the frequency of licking/grooming and arched-back nursing was highly correlated ($r = -0.91$) across animals and thus we were able to define mothers according to both behaviors—High or Low licking/grooming–arched-back nursing (LG-ABN) mothers. For the sake of most of the studies described here, High and Low LG-ABN mothers were identified as females whose scores on both measures were ±1 SD above (High) or below (Low) the mean for their cohort.[49]

The critical question, of course, concerns the potential consequences of these differences in maternal behavior for the development of behavioral and neuroendocrine responses to stress. Indeed, if postnatal handling results in more modest behavioral and HPA responses to stress through effects on maternal behavior, then the adult offspring of animals reared by High LG-ABN mothers should resemble animals handled as neonates. As adults, the offspring of High LG-ABN mothers showed reduced plasma ACTH and corticosterone responses to acute stress by comparison to the adult offspring of Low LG-ABN mothers. The High LG-ABN offspring also showed significantly increased hippocampal glucocorticoid receptor mRNA expression, enhanced glucocorticoid negative feedback sensitivity, and decreased hypothalamic CRH mRNA levels. Moreover, the magnitude of the corticosterone response to acute stress was significantly correlated with the frequency of both maternal licking/grooming ($r = -0.61$) and arched-back nursing ($r = -0.64$) during the first week of life, as was the level of hippocampal glucocorticoid receptor mRNA and hypothalamic CRH mRNA expression (all r's > 0.70).[46]

HOW MIGHT MATERNAL CARE REGULATE GENE EXPRESSION IN THE OFFSPRING?

The handling paradigm provides a model for understanding the mechanisms by which environmental stimuli can regulate neural development and physiology. This model is somewhat unique since most paradigms involving alterations in perinatal environmental conditions focus on changes in either synapse formation or neuron survival[50] that ultimately result in effects at the level of morphology. In contrast, handling affects neurochemical differentiation in the hippocampus, specifically altering the sensitivity of hippocampal cells to corticosterone, via an effect on gluco-

corticoid receptor gene expression and thus receptor density. Such variations in neuronal differentiation underlie important individual differences in tissue sensitivity to hormonal signals and thus represent a biochemical basis for environmental "programming" of neural systems.

The handling effect on the development of glucocorticoid receptor density in the hippocampus shows the common characteristics of a developmental effect. First, there is a specific "critical period" during which the organism is maximally responsive to the effects of handling. Second, the effects of handling during the first 21 days of life on glucocorticoid receptor density endure throughout the life of the animal. Finally, there is substantial specificity to the handling effect. Handling alters the glucocorticoid, but not mineralocorticoid, receptor gene expression. Interestingly, glucocorticoid and mineralocorticoid receptors are co-expressed in virtually all hippocampal neurons. Thus, the handling effect on gene expression is specific.

Temporal Features of the Handling Effect

Handling during the first week of life is as effective as handling during the entire first three weeks of life in reducing adrenal steroid responses to stress[51] and in increasing hippocampal glucocorticoid receptor density.[52] Handling over the second week of life is less effective, whereas animals handled between days 15 and 21 do not differ from non-handled animals in glucocorticoid receptor binding. Thus, in terms of both HPA activity and glucocorticoid receptor binding, the sensitivity of the system to environmental regulation decreases progressively over the first three weeks of life. Moreover, in comparison to same-aged non-handled animals, handled animals exhibited significantly increased hippocampal glucocorticoid receptor density as early as day 7 of life and the magnitude of the effect did not increase thereafter.[52] Thus, glucocorticoid receptor binding capacity appears to be especially sensitive to environmental regulation during the first week of life. However, please note that these findings do not preclude the possibility of other periods of environmental regulation. Indeed, a so-called critical period must be defined not only in terms of the target outcome, but also by the relevant input stimulus. Hence, we assume that these findings suggest that the critical period for the effect of handling on glucocorticoid receptor gene expression occurs during the first week of life.

The Role of Thyroid Hormones

Handling during the first week of life activates the hypothalamic-pituitary-thyroid axis leading to increased levels of circulating thyroxine (T_4) and increased intracellular levels of the biologically more potent T_4 metabolite, triiodothyronine (T_3). The pituitary-thyroid axis is a major regulator of HPA development (see Ref. 53 for a review). Neonatal treatment with either T_4 or T_3 resulted in significantly increased glucocorticoid receptor binding capacity in the hippocampus in animals examined as adults.[54] Like the handling manipulation, neither T_4 nor T_3 treatment affected hypothalamic or pituitary glucocorticoid receptor density. Moreover, administration of the thyroid hormone synthesis inhibitor, propylthiouracil (PTU), to handled pups for the first two weeks of life completely blocked the effects of handling on hippocampal glucocorticoid receptor binding capacity. These data are con-

sistent with the idea that the thyroid hormones might mediate, in part at least, the effects of neonatal handling on the development of the forebrain glucocorticoid receptor system.

Systemic injections of neonatal rat pups represent a rather crude manipulation, particularly procedures involving thyroid hormones. While these data might implicate the thyroid hormones, there is no indication that the hippocampus is actually the critical site of action. To examine whether thyroid hormones might act directly on hippocampal cells we used an *in vitro* system, involving primary cultures of dissociated hippocampal cells.[55] The hippocampal cells were taken from embryonic rat pups (E20) and beginning on the fifth day following plating the cultures were exposed to 0, 1, 10, or 100 nM T_3. These cells exhibit both mineralocorticoid and glucocorticoid receptor binding.[56] Indeed, both receptors as well as their mRNAs can be detected using material from a 60-mm dish. The results of several experiments have failed to detect any effect of thyroid hormones on glucocorticoid receptor density in cultured hippocampal cells. These *in vitro* data suggest that (1) the effects of the thyroid hormones on the glucocorticoid receptor binding occur at some site distal to the hippocampal cells or (2) thyroid hormones interact at the level of the hippocampus with some other hormonal signal that is obligatory for the expression of the thyroid hormone effect.

The Role of Serotonin

Thyroid hormones have pervasive effects throughout the developing central nervous system (CNS) and one such effect involves the regulation of central serotonergic neurons.[57] Thyroid hormones increase serotonin (5-HT) turnover in the hippocampus of the neonatal rat. [58] Handling also increases hippocampal 5-HT turnover[58,59] and thus both manipulations increase serotonergic stimulation of hippocampal neurons. There is also direct evidence for an effect of 5-HT on glucocorticoid receptor density in the neonatal rat. Lesioning of the raphe 5-HT neurons with 5,7-dihydroxytryptamine (5,7-DHT) dramatically reduces the ascending serotonergic input into the hippocampus. Rat pups administered 5,7-DHT on the second day of life showed reduced hippocampal glucocorticoid receptor density as adults.[58] Interestingly, neonatal administration of 5,7-DHT produces only a transient effect, such that by adulthood 5-HT innervations to the hippocampus are restored. The effect of hippocampal glucocorticoid receptor levels, however, persists into adulthood. This finding suggests that the effect of 5-HT on hippocampal glucocorticoid receptor expression, like handling itself, is unique to the first week of life.

Serotonin significantly increases glucocorticoid receptor density in cultured hippocampal cells.[56,60] In hippocampal cells cultured in the presence of increasing concentrations of 5-HT, there was a twofold increase in glucocorticoid receptor binding. The effect of 5-HT was dose related, with an EC_{50} of 4-5 nM and a maximal effect achieved at 10 nM concentrations that require a four-day treatment period. Shorter periods of exposure were ineffective, suggesting that the effect of 5-HT involves the increased synthesis of receptors. In support of this idea, we found that the effect of 5-HT on glucocorticoid receptor density in cultured hippocampal cells is blocked by either actinomycin-D or cycloheximide and is paralleled by an increase in glucocorticoid receptor mRNA levels.

The effect of 5-HT on glucocorticoid receptor expression occurs uniquely in the neuronal cell population. We found no effect of 5-HT on glucocorticoid receptor binding in hippocampal glial-enriched cell cultures. This finding is not surprising, since our initial studies were performed with cultures composed largely (~85%) of neuron-like cells.[56] Moreover, the composition of the cultures is unaffected by 5-HT treatment. We also examined the potential involvement of the glial cells by using a conditioned-medium experiment in which glial-enriched cultures were treated for five days with 5-HT and the medium was then used to feed neuronal cultures. This procedure had no effect on glucocorticoid receptor density, suggesting that the effect was not due to a 5-HT–induced glial secretory product.

The effects of 10 nM 5-HT on glucocorticoid receptor density in cultured hippocampal cells are completely blocked by the 5-HT_2 receptor antagonists, ketanserin and mianserin.[56,60] Moreover, the 5-HT_{2A} agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), 3-trifluoromethyl-phenylpiperazine monohydrochloride (TFMPP), and quipazine were also effective in increasing glucocorticoid receptor binding in hippocampal culture, although not as effective as 5-HT. Selective agonists or antagonists of the 5-HT_{1A} or 5-HT_3 receptors have no effect on glucocorticoid receptor binding. Using $^{125}I_7$-amino-8-iodo-ketanserin as radioligand, we found high-affinity 5-HT_{2A} binding sites in our cultured hippocampal cells.

We then examined the nature of the secondary messenger systems involved in this serotonergic effect on glucocorticoid receptor binding. Mitchell and colleagues[56] found that low nanomolar concentrations of 5-HT (EC_{50} = 7 nM) produce a fourfold increase in cAMP levels in cultured hippocampal cells, with no effect on cGMP levels. This increase in cAMP is blocked by ketanserin and at least partially mimicked by quipazine, TFMPP, and DOI. Indeed, there is a strong correlation (~0.97) between the effects of these 5-HT receptor agonists on cAMP and glucocorticoid receptor levels.

Treatment with the stable cAMP analogue, 8-bromo-cAMP or with 10 μM forskolin produces a significant increase in glucocorticoid receptor density in cultured hippocampal neurons.[60] The effect of 8-bromo-cAMP is concentration related, and the maximal effect of 8-bromo-cAMP (~190%) is comparable to that for 5-HT (~200%). Interestingly, as with 5-HT, the effects of 8-bromo-cAMP on glucocorticoid receptor mRNA levels and receptor density are not apparent until at least four days of treatment.

Taken together, these findings suggest that changes in cAMP concentrations may mediate the effects of 5-HT on glucocorticoid receptor synthesis in hippocampal cells. We[60] also found that the cyclic nucleotide–dependent protein kinase inhibitor, H8, completely blocked the effects of 10 nM 5-HT on glucocorticoid receptor binding in hippocampal cell cultures. In contrast, the protein kinase C inhibitor, H7, had no such effect. These data suggest that activation of protein kinase A is involved in the serotonergic regulation of hippocampal glucocorticoid receptor development.

These studies involve effects on intact cells with long incubation periods, and there is ample possibility for an interaction between second messenger systems. This issue also arises because the 5-HT_{2A} receptor is linked not to cyclic nucleotide, but phospholipase C–related second messenger systems. In both *in vivo* and *in vitro* studies, 5-HT_{2A} agonists increase both diacylglycerol (DAG) levels and inositol phosphate (IP) metabolism (notably IP_1) within hippocampal membranes.[61] There are numerous examples in the literature of such "crosstalk" between second messen-

ger systems and the stimulation of IP metabolism via phorbol esters alters cAMP levels.[62] However, in contrast to other compounds, such as glutamate or carbachol, we found that stimulation of IP metabolism by 5-HT in hippocampal slices was rather modest in animals during the first week of life.[63] Interestingly, the effect of 5-HT on IP metabolism in hippocampal slices is decreased in the handled animals on postnatal day 7, while the stimulation of DAG is slightly enhanced. However, the overall pattern of 5-HT stimulation is weak. This may be due to differences in receptor coupling at this time of life. In neonatal rat hippocampi the stimulation of IP metabolism occurred via 5-HT_{2C} and not 5-HT_{2A} receptors during the first weeks of life.[64] Since there is little 5-HT_{2C} receptor expression in dorsal hippocampus this may explain the weak stimulation of phospholipase C–related second messenger systems.

These data suggest that 5-HT directly stimulates cAMP formation in hippocampal neurons. This idea is not easy to reconcile with the involvement of a 5-HT_{2A} receptor. However, a number of 5-HT receptors have been cloned and these receptors directly stimulate adenylyl cyclase activity. These include the 5-HT_4, 5-HT_6, and 5-HT_7 receptors.[65] The mRNAs for each of these receptors is expressed in rat hippocampus. Moreover, the 5-HT_7 receptor binds ketanserin with high affinity. Interestingly, antidepressants increase glucocorticoid receptor mRNA in cortical and hippocampal cell cultures.[66,67] Both the 5-HT_6 and 5-HT_7 receptors bind various antidepressants with high affinity and the 5-HT_7 receptor shows a high affinity for ketanserin.

To examine the potential involvement of the 5-HT_7 receptor in mediating the increase in glucocorticoid receptor levels, we[68] measured receptor expression in cultured hippocampal neurons after treatment with 10 mM 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 seven days. All treatments resulted in an increase in glucocorticoid receptor levels. The effect of 5-CT on glucocorticoid receptor expression was blocked by methiothepin. Likewise, 5-CT produced a significant increase in cAMP levels and the effect was blocked by methiothepin. Pindolol, which binds to the 5-HT_{1A} but not the 5-HT_7 receptor, had little effect. These results further implicate the 5-HT_7 receptor. The increase in glucocorticoid receptor expression is also mimicked with 5-methoxytryptamine (5-MeOT), an effect blocked with methiothepin as well as H8, a PKA inhibitor. Over the course of these studies we found that other serotonergic agonists (quipazine, TFMPP, DOI) could partially mimic the 5-HT effect on glucocorticoid receptor levels; this, however, was the first evidence that a more selective serotonergic agonist, 5-CT, could fully mimic the 5-HT effect. This observation is consistent with the idea that the effect of 5-HT on glucocorticoid receptor expression in hippocampal neurons is mediated by a 5-HT_7 receptor via activation of cAMP.

Activation of cAMP pathways can regulate gene transcription through effects on a number of transcription factors, including of course the cAMP-response element binding protein (CREB) through an enhanced phosphorylation of CREB. Phospho-CREB (pCREB) regulates gene transcription through pathways that involve the cofactor, CREB-binding protein (CBP). To further examine the relevant signal transduction pathway, CBP expression was investigated by Western blot analysis. Primary hippocampal cell cultures treated with 10 mM 8-bromo cAMP, 50 nM 5-CT, and 100 nM 5-HT all showed a significant increase CBP expression. Furthermore, the profile of pCREB was similar to CBP. Treatment of primary hippocampal cell cultures with 50 nM 5-CT resulted in a significant increase in phosphorylation of CREB.

In Vivo *5-HT Effects on Glucocorticoid Receptor Expression*

Our *in vivo* studies[59] of 5-HT activity provide some insight into why the hippocampus is selectively affected by handling. In rat pups handled for the first seven days of life, and sacrificed immediately following handling on postnatal day 7, 5-HT turnover was significantly increased in the hippocampus, but not in the hypothalamus or amygdala (regions where handling has no effect on glucocorticoid receptor density). These data suggest that handling selectively activates certain ascending 5-HT pathways and that this effect underlies the sensitivity of this receptor system in specific brain regions to regulation by environmental events during the first week of life.

Clearly,one concern here is the relationship between our *in vitro* results and the *in vivo* condition. Thus, it is reassuring that effects of postnatal handling of rat pups on hippocampal glucocorticoid receptor binding are blocked by concurrent administration of ketanserin.[58] Moreover, ketanserin treatment also blocked the effects of T_3 on hippocampal glucocorticoid receptor expression.[69] This finding also supports the idea that thyroid hormones mediate the handling effect by serving to increase 5-HT activity. We also examined the effects of handling on cAMP levels in hippocampal tissue in neonatal rats and found that handling stimulates a fourfold increase in cAMP levels.[70] These increases in cAMP are almost completely abolished by concurrent treatment with either ketanserin or the thyroid hormone synthesis inhibitor, PTU. Thus, to date the results from these *in vivo* studies certainly appear consistent with our earlier *in vitro* experiments.

The regulation of gene transcription by cAMP[71–78] is mediated by various transcription factors including cyclic nucleotide response element binding proteins (CREBs), cyclic nucleotide response element binding modulators (CREMs), most of which seem to be antagonists for CREBs, and the activating transcription factor family (ATF-1, ATF-2, ATF-3). In addition to the CREB/CREM-ATF family, nerve growth factor–inducible factors (NGFI-A and NGFI-B) as well as activator protein-2 (AP-2) are inducible by cAMP.[73,79] The promoter region of the human and mouse glucocorticoid receptor gene has been cloned and at least partially sequenced[80,81] and contains numerous binding sites for most of these transcription factors, providing a mechanism whereby cAMP might increase glucocorticoid receptor expression.

We[70] used a variety of techniques to study potential handling-induced changes in the expression of these transcription factors in neonatal rat hippocampus. Handling resulted in no change in NGFI-B mRNA expression, a significant (i.e., two- to three-fold) increase in AP-2 mRNA expression, and a very substantial (i.e., eight- to ten-fold) increase in NGFI-A mRNA levels. The increase in NGFI-A expression occurred across all hippocampal cell fields and in virtually every neuron. The increase in AP-2 and NGFI-A mRNAs is apparent immediately following the termination of handling, persists for at least three hours, and is associated with an increase in both AP-2 and NGFI-A immunoreactivity, indicating that the increase in mRNA expression is reflected in changes in protein levels. The handling effects on both NGFI-A and AP-2 expression are blocked by ketanserin or PTU.

The challenge at this point is to define the molecular targets for the early environmental effects. First, we are assuming that one target for regulation is the promoter region of the glucocorticoid receptor gene. We[82] 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 conse-

quence of alternative splicing of potentially 11 different exon 1 sequences. 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. From the 10 alternate exon 1 sequences we identified by 5′ -RACE, four correspond to alternative exons 1 sequence previously identified in mouse—exons 1_1, 1_5, 1_9, and 1_{10}.[81,83] 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 demonstrated significant levels of six alternative exon 1 sequences *in vivo* in the rat, with differential expression in the liver, hippocampus, and thymus presumably reflecting tissue-specific differences in promoter activity. Two of the alternative exon 1 sequences (exon 1_6 and 1_{10}) were expressed in all tissues examined, together present in 77–87% of total glucocorticoid mRNA. The remaining glucocorticoid receptor transcripts contained tissue-specific alternative first exons. Hippocampal RNA contained significant levels of the minor exon 1_5-, 1_7-, and 1_{11}-containing glucocorticoid receptor mRNA variants that were expressed at either low or undetectable levels in liver and thymus.

In transient transfection experiments, a construct encoding the whole CpG island of the glucocorticoid receptor gene, including eight of the alternate exons 1 and the splice acceptor site within the intron 5′ of exon 2, fused to a luciferase reporter gene within exon 2, exhibited substantial promoter activity in all cell lines tested. 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.

Promoter activity was also associated with particular regions of the CpG island, where the fusion to luciferase was made within specific exon 1 sequences. In these cases, no splice acceptor site is available within the luciferase gene, and a transcriptional fusion is generated between the specific exon 1 and the luciferase reporter; luciferase activity therefore reflects transcription through the specific exon 1. Relative activity of these constructs in different cell types was similar with one notable exception, the exon 1_7 promoter sequence ($P1_7$). Interestingly, $P1_7$, fused to luciferase within exon 1_7, had the highest activity of any single promoter construct in B103 and C6 cells, both of which are CNS derived. The activity of this construct was low in hepatic cells, in which $P1_6$ and $P1_{10}$ had the highest activity. *In vivo*, glucocorticoid receptor mRNA transcripts containing exon 1_7 were present at significant levels in the hippocampus, but absent from the 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.

Interestingly, tissue-specific alternative exon 1_7 usage was altered by postnatal handling that, of course, increases glucocorticoid receptor expression in the hippocampus. Handling selectively elevated glucocorticoid receptor mRNA containing exon 1_7; there was, for example, no effect on exon 1_{10}. Predictably, maternal care also affected the expression of glucocorticoid receptor splice variants. Variants containing the exon 1_7 sequence were significantly increased in the adult offspring of High LG-ABN mothers.

Serotonin appears crucial in mediating the effects of neonatal handling upon glucocorticoid receptor expression within the hippocampus. The transcription factors

NGFI-A and AP-2 are implicated in the induction of glucocorticoid receptor in the hippocampus after handling or following 5-HT treatment. A sequence in the human glucocorticoid receptor gene that binds AP-2 *in vitro*[84] is completely conserved in the rat glucocorticoid receptor gene (at 22718).[82] Additionally, within the CpG island, the glucocorticoid receptor gene contains 16 GC boxes (GGGCGG), including a sequence exactly matching the consensus binding site for the family of zinc-finger proteins that includes NGFI-A[85] immediately upstream of exon 1_7. Thus, increases in AP-2 and NGFI-A induced by neonatal handling could increase transcription from a promoter adjacent to exon 1_7, leading to increased glucocorticoid receptor mRNA. In previous studies we found that handling increased the binding of both NGFI-A and AP-2 to a promoter sequence for the human glucocorticoid receptor containing consensus sequences for both transcription factors (Weaver and colleagues, unpublished observations).

So how do these effects result in the long-term differentiation of hippocampal neurons? There are two very intriguing features of the 5-HT effect that bear directly on the question of the hippocampal cell cultures as a model for neural differentiation. First, the effects of 5-HT on glucocorticoid receptor levels in hippocampal cell cultures are restricted to the first three weeks in culture. Thus, cultures treated with 10 nM 5-HT for seven days at any time during the first three weeks in culture show a significant increase in glucocorticoid receptor density; however, the effect is lost after this point. Cultures treated with 10 nM 5-HT for seven days during the third to fourth week following plating show no increase in glucocorticoid receptor binding. Second, and most exciting, the increase in glucocorticoid receptor binding capacity following exposure to 10 nM 5-HT persists after 5-HT removal from the medium—for as long as the cultures can be studied there is a sustained increase in glucocorticoid receptor levels well past the removal of 5-HT from the medium. We have gone as long as 50 days and have seen no decrease in the magnitude of the 5-HT effect. Thus, the effect of 5-HT on glucocorticoid receptor density observed in hippocampal culture cells mimics the long-term effects of early environmental events.

Thus, we arrive at the most interesting feature of these effects: the finding that these effects persist well beyond the period of the treatment. There are at least two possible explanations for this finding. First, *in vivo* the increase in 5-HT turnover associated with the handling procedure might be accompanied by an increase in 5-HT innervation of the hippocampus, which persists throughout the life of the animal. The increased 5-HT innervation could then serve to maintain the handling effect. This possibility seems unlikely. The effect in cell cultures persists in the absence of 5-HT in the medium. Moreover, handling does not permanently alter 5-HT innervation into the dorsal hippocampus using either electrochemical[59] or immunocytohistochemical (Desjardins and Meaney, unpublished observations) measures of 5-HT content.

HOW ARE THE EFFECTS OF MATERNAL CARE ON THE OFFSPRING SUSTAINED INTO ADULTHOOD?

Epigenomic Marking of the Exon 1_7 Glucocorticoid Receptor Promoter

DNA methylation is a stable, epigenomic mark at CpG dinucleotides, which is associated with stable variations in gene transcription.[86-88] Hypomethylation of CpG

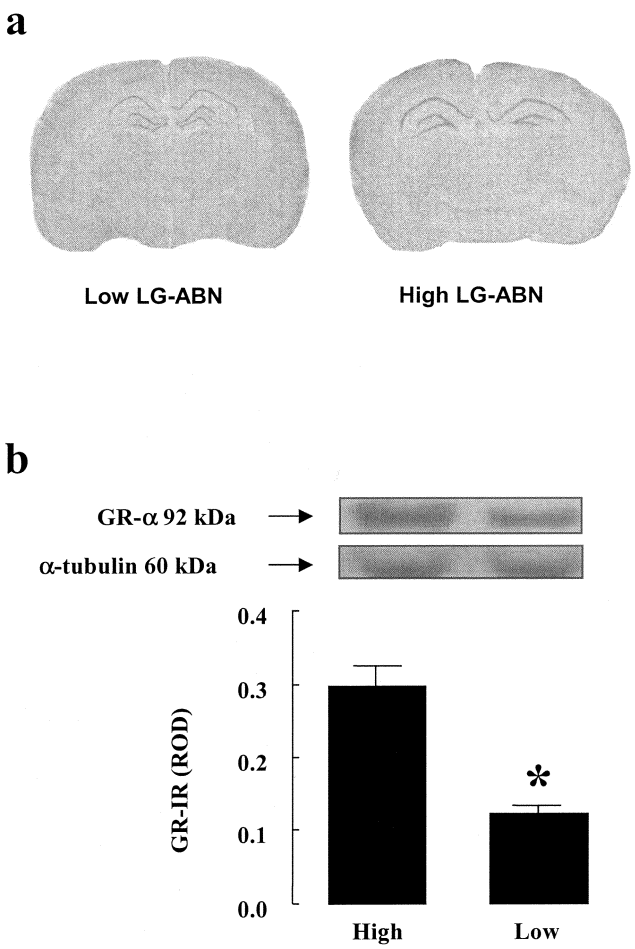


FIGURE 1. (**a**) A photomicrograph showing representative results of *in situ* hybridization studies of glucocorticoid receptor-α mRNA expression in the 90-day-old (adult) offspring of High and Low LG-ABN mothers. The results of earlier studies[46] reveal increased glucocorticoid receptor mRNA expression in the hippocampus of the adult offspring of High LG-ABN mothers. The *darkly labeled regions* in the coronal sections depicted above show the cell body regions of Ammon's horn and dentate gyrus of the dorsal hippocampus. (**b**) The *upper panel* reveals a representative Western blot illustrating glucocorticoid receptor-α and α-tubulin immunoreactivity (IR) in the 90-day-old (adult) offspring of High or Low LG-ABN mothers. Molecular weight markers (SeeBlue, Santa Cruz Biotech, Santa Cruz, CA) correspond to single major bands at 92 kDa and 60 kDa, with quantitative densitometric analysis relative optical density (ROD) of glucocorticoid receptor IR levels from samples (N = 5 animals/group). Western blot results indicate that hippocampal glucocorticoid receptor expression is greater in the adult offspring of High compared to Low LG-ABN mothers (*P <0.001).

dinucleotides of regulatory regions of genes correlates with active chromatin structure and transcriptional activity.[86,89] Thus, the methylation pattern is a stable signature of the epigenomic status of a regulatory sequence. We focused on the methylation state of the exon 1_7 glucocorticoid receptor promoter, which is activated in the hippocampus in offspring of High LG-ABN mothers. Glucocorticoid receptor gene expression is increased throughout the hippocampus in the adult offspring of High compared with Low LG-ABN mothers (FIG. 1).[40,90] We therefore examined the level of methylation across the entire exon 1_7 glucocorticoid receptor promoter sequence using the sodium bisulfite mapping technique. Sodium bisulfite treatment of DNA samples converts non-methylated cytosines to uracils; methylated cytosines are unaffected and the differences in methylation status are thus apparent on sequencing gels. In preliminary studies, we found significantly greater methylation of the exon 1_7 glucocorticoid receptor promoter sequence in the offspring of the Low LG-ABN mothers (FIG. 2). These findings are consistent with the hypothesis that maternal effects alter DNA methylation patterns in the offspring.

Two kinds of changes in DNA methylation are known to affect gene expression; regional, non–site-specific DNA methylation around a promoter[91] and site-specific methylation. To determine whether DNA methylation of specific target sites on the

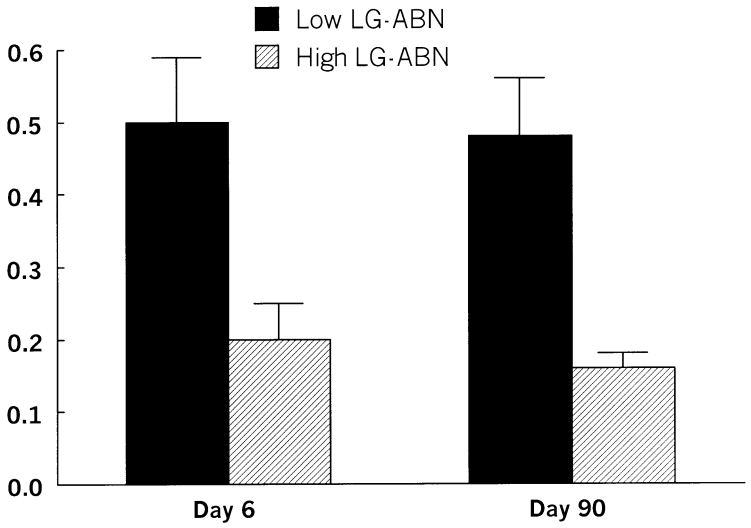


FIGURE 2. Global methylation pattern of the exon 1_7 glucocorticoid receptor promoter in 6-day-old (young pup) and 90-day-old (adult) offspring of High and Low LG mothers ($N = 4$ animals/group). The values are an average of the percentage methylation per cytosine for all 17 CpG dinucleotides in the two treatment groups (*$P < 0.0001$). Statistical analysis with a two-way ANOVA (Group × Age) of the degree of cytosine methylation across the entire exon 1_7 glucocorticoid receptor promoter revealed a highly significant Group effect $F = 24.581$, $P < 0.0001$.

glucocorticoid receptor promoter change in response to maternal care, we mapped the differences in methylation using the sodium bisulfite mapping technique,[92,93] focusing on a region around the NGFI-A consensus sequence within the exon 1_7 promoter. The results revealed significant differences in the methylation of specific regions of the exon 1_7 glucocorticoid receptor promoter sequence. For example, the cytosine within the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence is always methylated in the offspring of Low LG-ABN mothers and rarely methylated in the offspring of High LG-ABN dams.

Maternal Care and Glucocorticoid Receptor Promoter Methylation

While these findings suggest that specific sites in the exon 1_7 glucocorticoid receptor promoter are differentially methylated as a function of maternal behavior, such findings are merely correlational. To directly examine the relation between maternal behavior and DNA methylation changes within the exon 1_7 glucocorticoid receptor promoter, we 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 hours of birth.[47] Cross-fostering the biological offspring of High LG-ABN mothers or dams produced a pattern of exon 1_7 glucocorticoid receptor promoter methylation that was associated with the rearing mother. Most importantly, the cross-fostering procedure reversed the difference in methylation at specific cytosines. For example, the cytosine within the 5′ CpG dinucleotide (site 16) of the NGF I-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 (site 16) 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 was indistinguishable from that of the biological offspring of High LG-ABN mothers. Interestingly, cross-fostering did not have the same effect upon the methylation status of every CpG dinucleotide. For example, the CpG dinucleotide (site 12) 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 remained hypomethylated; whereas the CpG dinucleotide (site 12) 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 remained hypermethylated. Of course, it is tempting to speculate that when the AP-1 consensus sequence is demethylated the exon 1_7 glucocorticoid receptor promoter is transcriptionally active, whereas the methylation status of 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence controls the relative levels of transcriptional activity, acting as a dimmer switch for transcription upon loading of methyl groups. However, NGFI-A may bind to the cognate response element and transactivate the exon 1_7 glucocorticoid receptor promoter independent of the AP-1 consensus sequence methylation status. Regardless, 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. This is the first demonstration of a DNA methylation pattern established through a behavioral mode of programming. Parental imprinting, a well-established paradigm of inheritance of an epigenomic mark, requires germ-line transmission.[88]

Maternal Care–Driven Demethylation of the Exon 1_7 Glucocorticoid Receptor Promoter

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.[40,46] Thus, we wondered whether this period corresponds to the timing for the appearance of the difference in DNA methylation in the offspring. We used the sodium bisulfite mapping technique to map precisely the methylation status of the cytosines within the exon 1_7 glucocorticoid receptor promoter over multiple developmental time points (FIG. 3a-e). This analysis demonstrates that just before birth, on embryonic day 20, the entire 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 (FIG. 3b). 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.[94] 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 this glucocorticoid receptor promoter. Between postnatal day 1 and postnatal day 6, the period when differences in the maternal behavior of High and Low LG-ABN dams are apparent, differences in the status of methylation of the exon 1_7 glucocorticoid receptor develop between the two groups. For example, the NGFI-A response element 5′ CpG dinucleotide (site 16) is demethylated in the High, but not in the Low LG-ABN group (FIG. 5c). This is consistent with data from the cross-fostering experiment, which illustrated that the differences between the two groups developed following birth in response to maternal behavior. The group difference in CpG dinucleotide methylation then remains consistent through to adulthood (postnatal day 90; FIG. 3c–e). Interestingly, the CpG dinucleotide (site 12) of the AP-1 consensus sequence within the exon 1_7 glucocorticoid receptor promoter is similarly hypomethylated in the High LG-ABN offspring by postnatal day 6, and this hypomethylation is sustained into 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.[95]

The results of developmental time-line study are very intriguing. From postnatal day 6, the methylation patterns for each of the 17 individual CpG sites within the exon 1_7 glucocorticoid receptor promoter do not all remain at the exactly same frequency for each developmental time-point (compare FIG. 3c-e). This is consistent with the model that methylation, like most (if not all) biological processes, is in a constant flux, but is stably maintained through a dynamic equilibrium. The developmental time-line may also help explain why the effect of maternal care on the hippocampal glucocorticoid receptor gene activity is not easily reversed when a High LG-ABN offspring is cross-fostered to a Low LG-ABN mother, in comparison to the

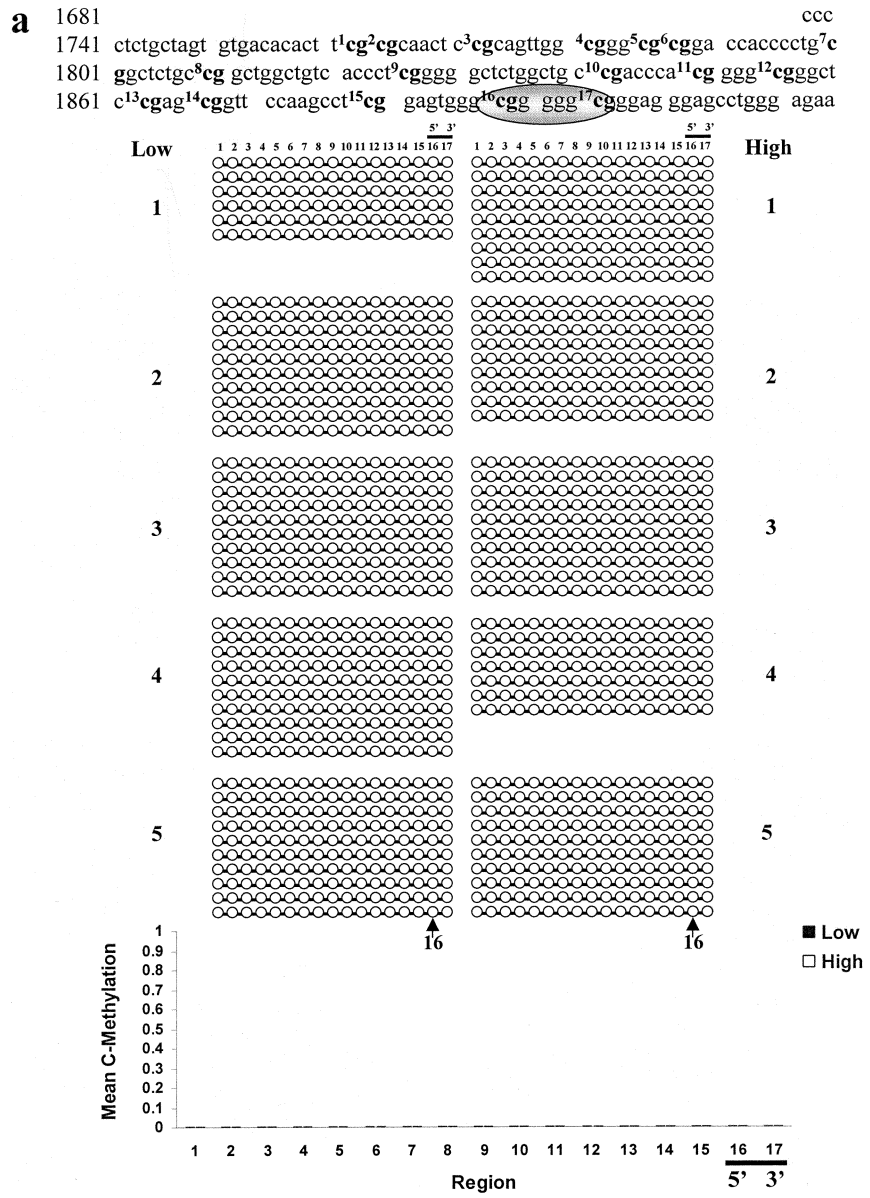
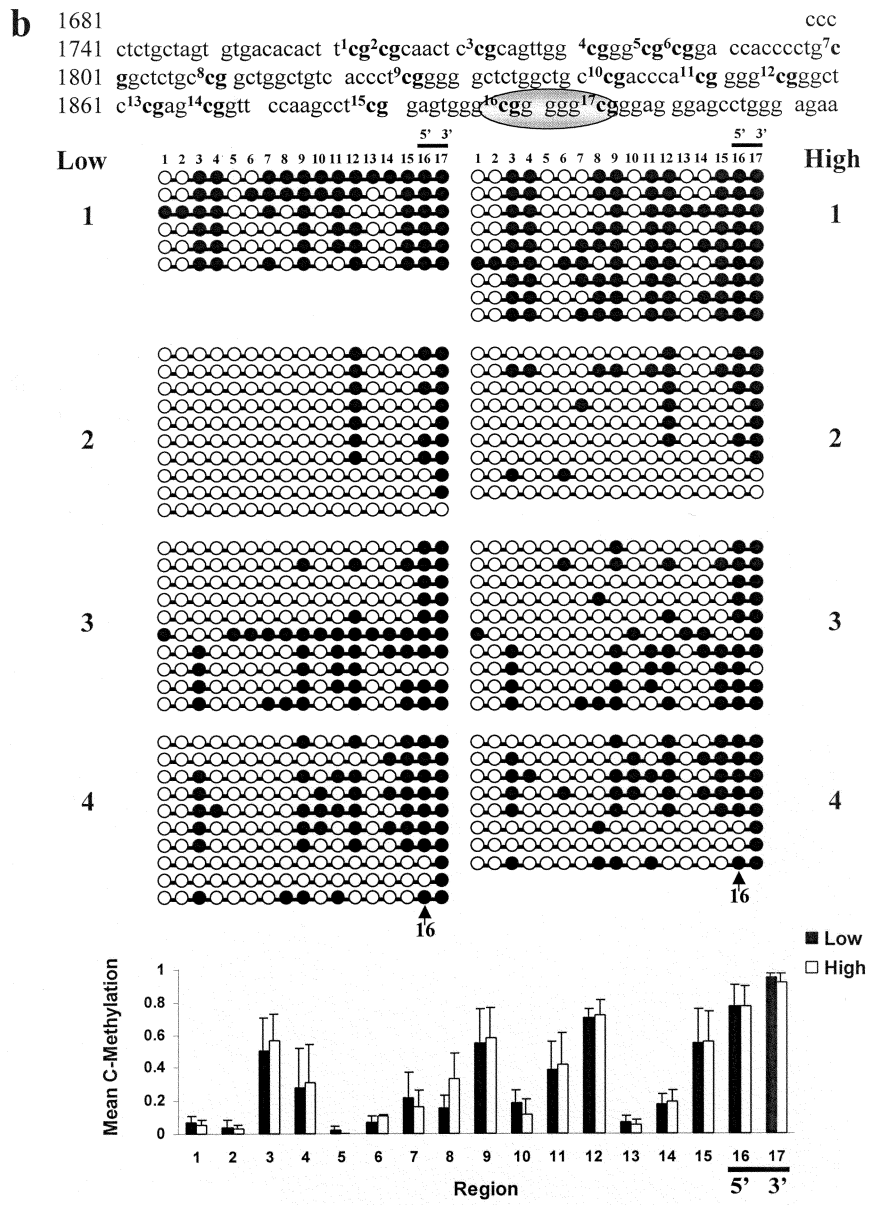
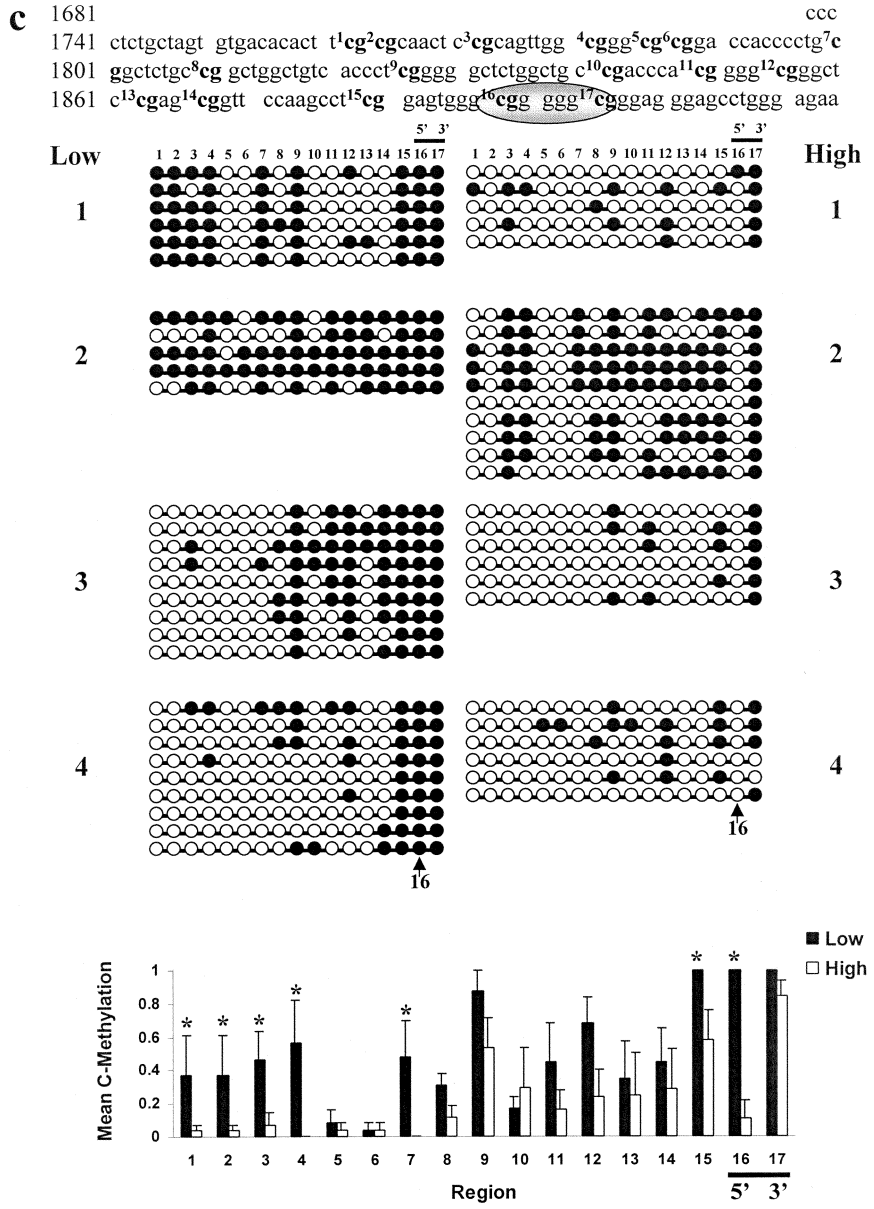


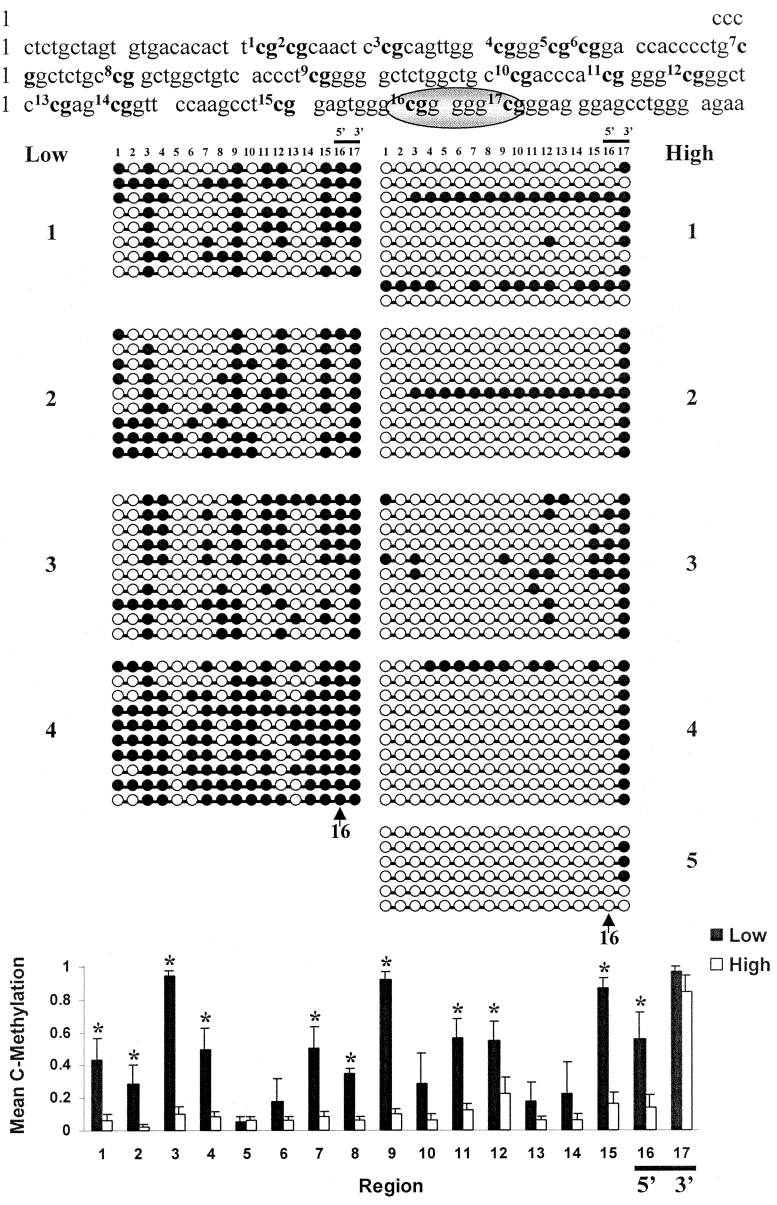
FIGURE 3. (**a**) Methylation patterns of the exon 1_7 glucocorticoid receptor promoter in the hippocampi of ED20 High and Low LG-ABN offspring ($N = 5$ animals/group). *Top panel* shows a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (*highlighted in bold*) and the NGFI-A binding region (*encircled*). *Middle panel* shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter re-



gion analyzed by sodium bisulfite mapping (6–10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region are labeled 1–17, highlighting the two CpG dinucleotides site 16 (5′) and site 17 (3′) within the NGFI-A binding region (*black line*). *Black circles* indicate methylated cytosines and *white circles* indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5′) is again highlighted (*black arrow*). *Bottom panel* shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region from embryonic High and Low LG-ABN



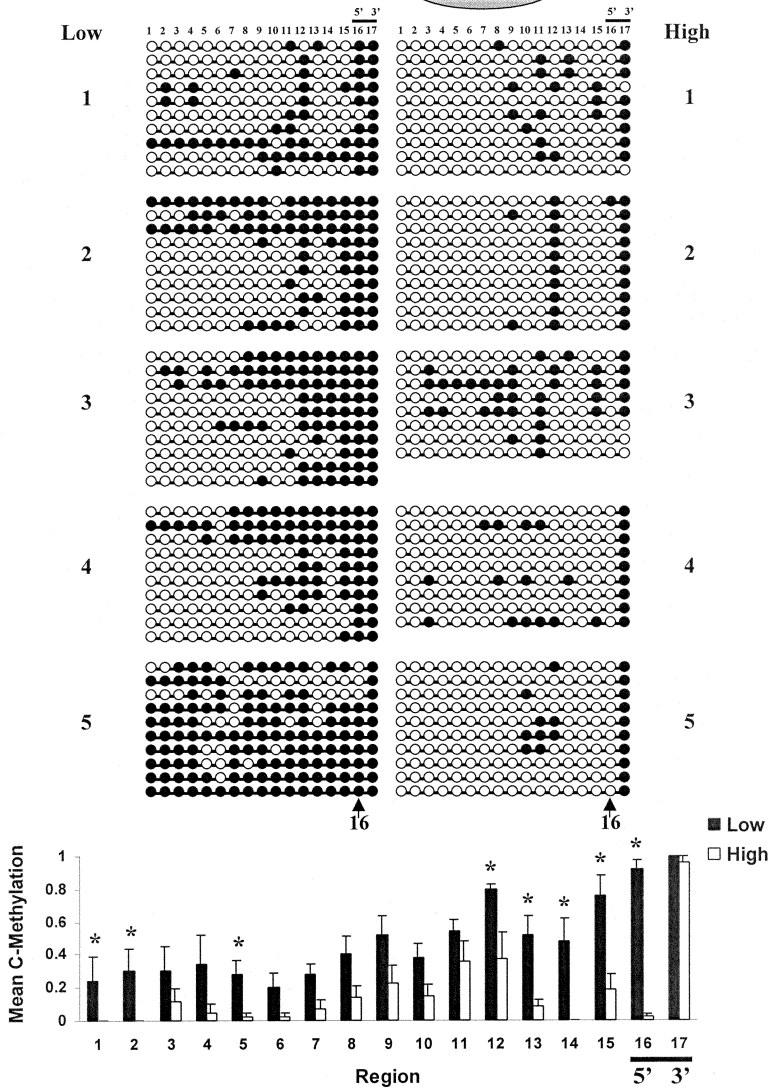
offspring. (**b**) Methylation patterns of the exon 1_7 glucocorticoid receptor promoter in the hippocampi of 1-day-old (within one hour after birth) High and Low LG-ABN offspring (N = 4 animals/group). *Top panel* shows a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (highlighted in bold) and the NGFI-A binding region (*encircled*). *Middle panel* shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 glucocorticoid



receptor promoter region analyzed by sodium bisulfite mapping (6–10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region are labeled 1–17, highlighting the two CpG dinucleotides site 16 (5′) and site 17 (3′) within the NGFI-A binding region (*black line*). *Black circles* indicate methylated cytosines and *white circles* indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5′) is again highlighted (*black arrow*). *Bottom panel* shows methylation analysis of the 17 CpG

e

1681 ccc
1741 ctctgctagt gtgacacact t[1]**cg**[2]**cg**caact c[3]**cg**cagttgg [4]**cg**gg[5]**cg**[6]**cg**ga ccacccctg[7]**c**
1801 **g**gctctgc[8]**cg** gctggctgtc accct[9]**cg**ggg gctctggctg c[10]**cg**accca[11]**cg** ggg[12]**cg**ggct
1861 c[13]**cg**ag[14]**cg**gtt ccaagcct[15]**cg** gagtggg[16]**cg**g ggg[17]**cg**ggag ggagcctggg agaa



dinucleotides of the exon 1_7 glucocorticoid receptor promoter region from postnatal day-1 High and Low LG offspring. The two-way ANOVA (Group × Region) revealed a highly significant effect of Region [F =10.337, P <0.0001]. (**c**) Methylation patterns of the exon 1_7 glucocorticoid receptor promoter in the hippocampi of 6-day-old (young pup) High and Low LG-ABN offspring (N = 4 animals/group). *Top panel* shows a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (*highlighted in bold*)

substantial increase in hippocampal glucocorticoid receptor gene activity observed when a Low LG-ABN offspring is cross-fostered to a High LG-ABN mother. DNA methylation is a thermodynamically slower process in comparison to active demethylation. This implies that the hippocampal exon 1_7 glucocorticoid receptor promoter, within the Low LG-ABN mother's biological offspring, becomes stripped of CpG methylation by activity-dependent processive demethylation, resulting from the in-

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and the NGFI-A binding region (*encircled*). *Middle panel* shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region analyzed by sodium bisulfite mapping (5–10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region are labeled 1–17, highlighting the two CpG dinucleotides site 16 (5′) and site 17 (3′) within the NGFI-A binding region (*black line*). *Black circles* indicate methylated cytosines and *white circles* indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5′) is again highlighted (*black arrow*). *Bottom panel* shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region of the glucocorticoid receptor from postnatal day-6 High and Low LG-ABN offspring $*P < 0.05$; $*P < 0.0001$ for site 16 (5′) lying within the NGFI-A binding region. The two-way ANOVA (Group × Region) revealed a highly significant effect of both Group [$F = 32.569$, $P < 0.0001$] and Region [$F = 5.353$, $P < 0.0001$]. The Group × Region interaction effect was not significant [$F = 1.265$, $P = 0.057$]. (**d**) Methylation patterns of the exon 1_7 glucocorticoid receptor promoter in the hippocampi of 21-day-old (weaning age) High and Low LG-ABN offspring ($N = 4$–5 animals/group). *Top panel* shows a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (*highlighted in bold*) and the NGFI-A binding region (*encircled*). *Middle panel* shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region analyzed by sodium bisulfite mapping (6–10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region are labeled 1–17, highlighting the two CpG dinucleotides site 16 (5′) and site 17 (3′) within the NGFI-A binding region (*black line*). *Black circles* indicate methylated cytosines and *white circles* indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5′) is again highlighted (*black arrow*). *Bottom panel* shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region from postnatal day 21 High and Low LG-ABN offspring ($*P < 0.05$). The two-way ANOVA (Group × Region) revealed a highly significant effect of both Group [$F = 150.450$, $P < 0.0001$] and Region [$F = 12.474$, $P < 0.0001$], as well as a significant Group × Region interaction effect [$F = 4.223$, $P < 0.0001$]. (**e**) Methylation patterns of the exon 1_7 glucocorticoid receptor promoter in the hippocampi of 90-day-old (adult) High and Low LG-ABN offspring ($N = 5$ animals/group). *Top panel* shows a sequence map of the exon 1_7 glucocorticoid receptor promoter including the 17 CpG dinucleotides (*highlighted in bold*) and the NGFI-A binding site (*encircled*). *Middle panel* shows bead-on-string representation of the cytosine methylation status of each of the 17 individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region analyzed by sodium bisulfite mapping (8–10 clones sequenced/animal). The individual CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region are labeled 1–17, highlighting the two CpG dinucleotides site 16 (5′) and site 17 (3′) within the NGFI-A binding region (*black line*). *Black circles* indicate methylated cytosines and *white circles* indicate non-methylated cytosines. The CpG dinucleotide at site 16 (5′) is again highlighted (*black arrow*). *Bottom panel* shows methylation analysis of the 17 CpG dinucleotides of the exon 1_7 glucocorticoid receptor promoter region from postnatal day-90 High and Low LG-ABN offspring $*P < 0.05$; $*P < 0.0001$ for site 16 (5′) lying within the NGFI-A binding region. The two-way ANOVA (Group × Region) revealed a highly significant effect of Group [$F = 104.782$, $P < 0.0001$] and Region [$F = 11.443$, $P < 0.0001$], as well as a significant Group × Region interaction effect [$F=2.321$, $P<0.01$].

tense tactile stimulation exerted toward the pups by the High LG-ABN foster mother. Whereas the hippocampal exon 1_7 glucocorticoid receptor promoter within the High LG-ABN mother's biological offspring passively gains CpG methylation (assuming some loss of methylation through maternal care by the biological High LG-ABN parent prior to cross-fostering), through a lack of stimulation of processive demethylation by the Low LG-ABN adoptive parent. The fact that High and Low LG-ABN offspring differ on epigenomic control of gene activity leaves the reader to speculate upon why the Low LG-ABN offspring are so plastic in response to the foster, High LG-ABN mother.

Site-Specific Methylation of the Cytosine within the 5′ CpG Dinucleotide (Site 16) of the NGFI-A Response Element Blocks Transcription Factor Binding

The next question concerns the functional importance of such differences in methylation. DNA methylation affects gene expression either by attracting methylated DNA binding proteins to a densely methylated region of a gene[96] or by site-specific interference with the binding of a transcription factor to its recognition element.[97] Our data showing site-specific demethylation of the cytosine within the 5′ CpG dinucleotide (site 16) of the NGFI-A response element (FIG. 3c) 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, we determined the *in vitro* binding of increasing concentrations of purified recombinant NGFI-A protein[98] 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 (FIG. 4a) bearing the NGFI-A binding site that was either (1) non-methylated, (2) methylated in the 3′ CpG site, (3) methylated in the 5′ CpG site, (4) methylated in both sites, or (5) mutated at the two CpGs with an adenosine replacing the cytosines. NGFI-A formed a protein-DNA complex with the non-methylated oligonucleotide (FIG. 4b, lanes 2-4), while the protein was unable to form a complex with either a fully methylated sequence or a sequence that was methylated at the 5′ CpG site (FIG. 4b, lanes 10–12, 14–16). Partial activity was seen with the sequence methylated at the 3′ CpG site (FIG. 4b, lanes 6-8). The specificity of the protein-DNA interaction is indicated by the fact that the recombinant protein fails to form a complex with the mutated NGFI-A response element, even at high protein concentrations (36 pM) (FIG. 4b, lanes 18-20). This difference in binding was confirmed by competition experiments (FIG. 4c). NGFI-A recombinant protein was incubated with a labeled, non-methylated oligonucleotide in the presence of 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. As expected, the non-methylated oligonucleotide competed with the labeled oligonucleotide protein-DNA complex (FIG. 4c, lane 7). The specificity of the protein-DNA interaction is indicated by the fact that the mutated oligonucleotide is unable to compete away the labeled oligonucleotide protein-DNA complex (FIG. 4c lanes 17–19). Neither the oligonucleotide methylated in both the 3′ and 5′ CpGs nor the 5′ CpG methylated oligonucleotide were able to compete (FIG. 4c, lanes 11–16). Importantly, the 3′ CpG methylated oligonucleotide, which mimics the sequence observed in the off-

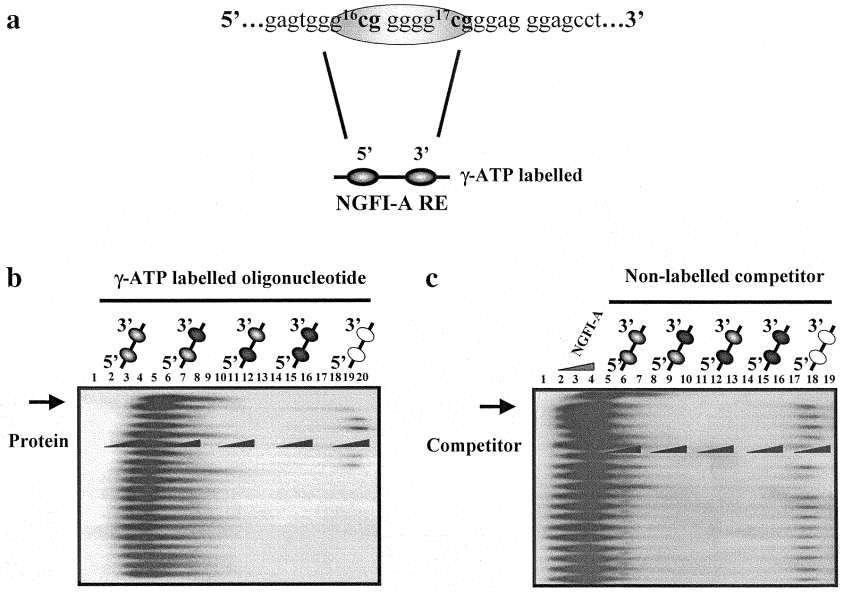

FIGURE 4. (**a**) The exon 1_7 glucocorticoid receptor promoter sequence with the NGFI-A binding region (*encircled*). Beneath is a bead-on-string representation of a synthesized radio-labeled oligonucleotide probe, highlighting the two CpG dinucleotides [*ovals* represent the cytosines at site 16 (5′) and site 17 (3′)] within the NGFI-A binding region (response element, RE). (**b**) EMSA analysis of protein-DNA complex formation between recombinant purified NGFI-A protein and radiolabeled oligonucleotide (**a**) bearing the NGFI-A response element containing differentially methylated cytosines within the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides. Non-methylated cytosines are represented by *gray ovals*, methylated cytosines are shown as *black ovals*, and *white ovals* are mutated CpG dinucleotides with an adenosine replacing the cytosine. The presence of increasing amounts of purified NGFI-A protein (9 pM, 18 pM, or 36 pM) is indicated by the *black triangle*. The *black arrow* indicates the shift in labeled oligonucleotide mobility. Lane 1: free oligonucleotide non-methylated at either dinucleotide. Lanes 2-4: non-methylated oligonucleotide with an increasing amount of NGFI-A. Lane 5: free oligonucleotide methylated at the 3′ CpG dinucleotide. Lanes 6-8: oligonucleotide methylated at the 3′ CpG dinucleotide with an increasing amount of NGFI-A. Lane 9: free oligonucleotide methylated at the 5′ CpG dinucleotide. Lanes 10-12: oligonucleotide methylated at the 5′ CpG dinucleotide with an increasing amount of NGFI-A. Lane 13: free oligonucleotide methylated at both 5′ and 3′ CpG dinucleotides. Lanes 14-16: oligonucleotide methylated at both 5′ and 3′ CpG dinucleotides with an increasing amount of NGFI-A. Lane 17: free non-methylated oligonucleotide mutated with an adenosine replacing the cytosine in both the 5′ and 3′ CpG dinucleotides. Lanes 18-20: mutated non-methylated oligonucleotide with increasing amount of NGFI-A. Note, methylation of the cytosine within the 3′ CpG dinucleotide reduced binding at the higher levels of NGFI-A protein, while methylation of the cytosine within the 5′ CpG dinucleotide completely eliminated protein binding to the NGFI-A binding region (response element, RE). (**c**) EMSA analysis of competition of protein-DNA complex formation between NGFI-A protein and a radiolabeled oligonucleotide probe containing the NGFI-A response element (RE) (**a**) by an excess of non-labeled oligonucleotides containing differentially methylated cytosines within the 5′ and 3′ CpG dinucleotides of the NGFI-A response element (RE). Non-methylated cytosines are represented by *gray ovals*, methylated

spring of High LG-ABN mothers, exhibited substantial competition (FIG. 4c, lanes 8–10). The results indicate that while methylation of the cytosine within the 5′ CpG dinucleotide (site 16) reduced NGFI-A protein binding to the same extent as methylation in both CpG sites, methylation of the cytosine within the 3′ CpG dinucleotide (site 17) only partially reduced NGFI-A protein binding (FIG. 4a, b). These data support the hypothesis that methylation of the cytosine within the 5′ CpG dinucleotide (site 16) 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, potentially explaining the reduced glucocorticoid receptor gene transcription in the offspring of the Low LG-ABN mothers.

Forming a Mechanism for Environment-Driven Gene-Imprinting of Behavior

The defining question of early experience studies concerns the mechanism by which environmental effects occurring in early development are sustained into adulthood (i.e., "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 by which maternal behavior can sustain maternal effects into adulthood (see FIG. 5 for our proposed model). We show that forms of maternal care that increase tactile stimulation of the neonate (i.e., licking/grooming and arched-back nursing) result in a functional demethylation of a number of sites in the exon 1_7 glucocorticoid receptor promoter as well as increased acetylation of H3 histones and increased occupancy of the transcription factor NGFI-A on the exon 1_7 glucocorticoid receptor promoter, which is stably maintained into adulthood.[99]

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cytosines are shown as *black ovals*, and *white ovals* are mutated CpG dinucleotides with an adenosine replacing the cytosine. The presence of increasing amounts of purified NGFI-A protein (9 pM, 18 pM, or 36 pM) is indicated by the *grey triangle*. The presence of increasing amount of non-labeled oligonucleotide competitor (1:100 fold, 1:500 fold, or 1:1,000 fold) is indicated by the *black triangle*. The *black arrow* indicates the shift in labeled oligonucleotide mobility. Lane 1: free labeled non-methylated oligonucleotide. Lanes 2-4: labeled non-methylated oligonucleotide with increasing amount of NGFI-A. Lanes 5–7: labeled non-methylated oligonucleotide with increasing amount of non-labeled non-methylated oligonucleotide competitor. Lanes 8–10: labeled non-methylated oligonucleotide with increasing amount of non-labeled oligonucleotide competitor methylated at the 3′ CpG dinucleotide. Lanes 11–13: labeled non-methylated oligonucleotide with increasing amount of non-labeled oligonucleotide competitor methylated at the 5′ CpG dinucleotide. Lanes 14–16: labeled non-methylated oligonucleotide with increasing amount of non-labeled oligonucleotide competitor methylated at both 5′ and 3′ CpG dinucleotides. Lanes 17–19: labeled non-methylated oligonucleotide with increasing amount of non-labeled oligonucleotide competitor non-methylated but mutated with an adenosine replacing the cytosine in both the 5′ and 3′ CpG sites. Note that only the oligonucleotides with a non-methylated cytosine at the 5′ CpG dinucleotide of the NGFI-A binding region (response element, RE) showed effective competition. Methylation of the cytosine within the 5′ CpG dinucleotide completely eliminated the ability of the non-labeled oligonucleotides to compete for NGFI-A protein binding to the radiolabeled, non-methylated oligonucleotide sequence containing the NGFI-A binding region.

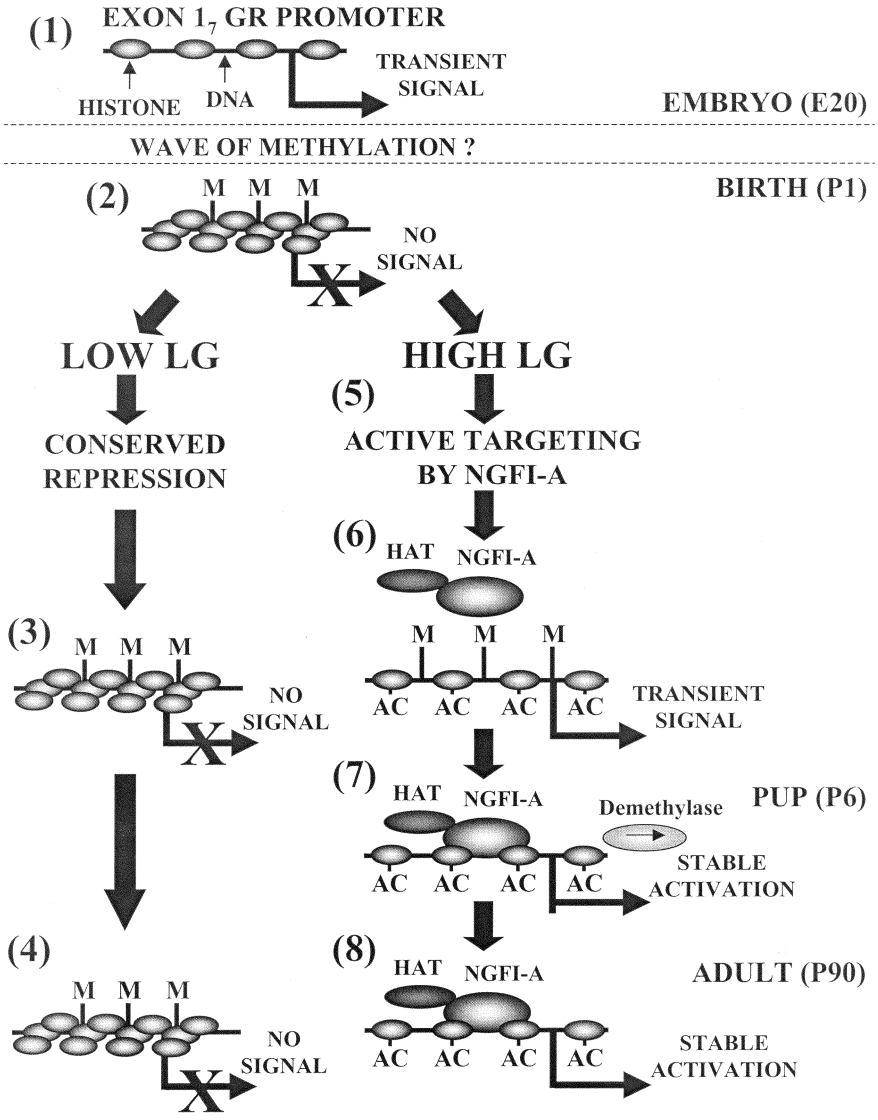

FIGURE 5. A model for environmental gene programming. (**1**) Prior to parturition the hippocampal exon 1_7 glucocorticoid receptor is entirely non-methylated (FIG. 3a). (**2**) Following birth, the hippocampal exon 1_7 glucocorticoid receptor becomes hypermethylated (FIG. 3b) and associated with tightly packed histones to form inactive chromatin. (**3**) In the absence of High tactile stimulation, the hippocampal exon 1_7 glucocorticoid receptor of the Low LG-ABN offspring remains hypermethylated and associated with the inactive chromatin, which endured into adulthood (**4**) (compare FIG. 3c-e). (**5**) High licking/grooming dams stimulate and maintain increased levels of activity-dependent gene expression in the offspring. The activity-dependent transcription factor NGFI-A actively targets its cognate response element within the hypermethylated hippocampal exon 1_7 glucocorticoid receptor.

Note that the effect of maternal care on glucocorticoid receptor expression is subtler (FIG. 1a, b) than the more pronounced effect on the methylation status of the 5′ CpG dinucleotide (site 16). However, in previous studies[82] we found evidence for the use of at least three promoters regulating hippocampal glucocorticoid receptor expression, suggesting that exon 1_7 is but one of the regulatory sequences determining glucocorticoid receptor expression within the hippocampus.

Environmental Variability Meets Epigenomic Predictability

Further studies are required to determine how maternal behavior alters the epigenomic status of the exon 1_7 glucocorticoid receptor promoter. In addition, the exact causal relationship between DNA methylation and other changes in the epigenomic status described here, such as altered histone acetylation and NGFI-A binding, remains unclear. Regardless of these as-yet-unanswered questions, our findings provide the first evidence that maternal behavior, early after birth, stably alters the epigenome of the offspring, providing a mechanism for the long-term effects of early experience on gene expression in the adult. These studies 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. Finally, 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,[7,100,101] 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. Maternal effects on defensive responses to threat are apparent in plants, insects, and reptiles. Such effects commonly follow from the exposure of the mother to the same or similar forms of threat and may represent examples where the environmental experience of the mother is translated through an epigenetic mechanism of inheritance into phenotypic variation in the offspring. Indeed, maternal effects could result in the transmission of adaptive responses across generations.[7,100,101] 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 on the fixed genome resulting in stable alterations in phenotype.

(**6**) Transcription factors commonly recruit histone modifying proteins, such as histone acetylase transferase (HAT). DNA methylation is associated with changes in chromatin activity states that gate accessibility of promoters to transcription factors through effects on histone acetylation (AC), a marker of active chromatin. Acetylation of the histone tails neutralizes the positively charged histones, which disrupts histone binding to negatively charged DNA and thus promotes transcription factor binding, resulting in transient exon 1_7 glucocorticoid receptor promoter activity. (**7**) Following processive DNA demethylation (compare FIG. 3b and c), the transcription factor NGFI-A can firmly bind to the demethylated response element (FIG. 4b, c) within the hippocampal exon 1_7 glucocorticoid receptor and allow stable transcription. The hippocampal exon 1_7 glucocorticoid receptor of the High LG-ABN offspring remains hypomethylated into adulthood (**8**), allowing stable transcription that is sustained throughout life (FIG. 1a, b).

REFERENCES

1. DENENBERG, V.H. 1964. Critical periods, stimulus input, and emotional reactivity: A theory of infantile stimulation. Psychol. Rev. **71:** 335–351.
2. DENENBERG, V.H. *et al.* 1967. Increased adrenocortical activity in the neonatal rat following handling. Endocrinology **81:** 1047–1052.
3. LEVINE, S. 1966. Infantile stimulation and adaptation to stress. Res. Publ. Assoc. Res. Nerv. Ment. Dis. **43:** 280–291.
4. LEVINE, S. 1957. Infantile experience and resistance to physiological stress. Science **126:** 405.
5. LEVINE, S. 1962. Plasma-free corticosteroid response to electric shock in rats stimulated in infancy. Science **135:** 795–796.
6. ZARROW, M.X., P.S. CAMPBELL & V.H. DENENBERG. 1972. Handling in infancy: increased levels of the hypothalamic corticotropin releasing factor (CRF) following exposure to a novel situation. Proc. Soc. Exp. Biol. Med. **141:** 356–358.
7. MEANEY, M.J. 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. **24:** 1161–1192.
8. CHROUSOS, G.P. & P.W. GOLD. 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. J. Am. Med. Assoc. **267:** 1244–1252.
9. AKANA, S.F. *et al.* 1992. Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. Endocrinology **131:** 57–68.
10. MCEWEN, B.S. & E. STELLAR. 1993. Stress and the individual. Mechanisms leading to disease. Arch. Intern. Med. **153:** 2093–2101.
11. SECKL, J.R. & M.J. MEANEY. 1993. Early life events and later development of ischaemic heart disease. Lancet **342:** 1236.
12. ADER, R. 1970. The effects of early experience on the adrenocortical response to different magnitudes of stimulation. Physiol. Behav. **5:** 837–839.
13. MEANEY, M.J. *et al.* 1989. Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology **50:** 597–604.
14. VIAU, V. *et al.* 1993. Increased plasma ACTH responses to stress in nonhandled compared with handled rats require basal levels of corticosterone and are associated with increased levels of ACTH secretagogues in the median eminence. J. Neurosci. **13:** 1097–1105.
15. BHATNAGAR, S. & M.J. MEANEY. 1995. Hypothalamic-pituitary-adrenal function in chronic intermittently cold-stressed neonatally handled and non handled rats. J. Neuroendocrinol. **7:** 97–108.
16. MEANEY, M.J. *et al.* 1991. Postnatal handling attenuates certain neuroendocrine, anatomical, and cognitive dysfunctions associated with aging in female rats. Neurobiol. Aging **12:** 31–38.
17. MEANEY, M.J. *et al.* 1992. Basal ACTH, corticosterone and corticosterone-binding globulin levels over the diurnal cycle, and age-related changes in hippocampal type I and type II corticosteroid receptor binding capacity in young and aged, handled and nonhandled rats. Neuroendocrinology **55:** 204–213.
18. LANDFIELD, P.W. & T.A. PITLER. 1984. Prolonged Ca^{2+}-dependent after hyperpolarizations in hippocampal neurons of aged rats. Science **226:** 1089–1092.
19. SAPOLSKY, R.M., L.C. KREY & B.S. MCEWEN. 1984. Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proc. Natl. Acad. Sci. USA **81:** 6174–6177.
20. KERR, D.S. *et al.* 1989. Corticosteroid modulation of hippocampal potentials: increased effect with aging. Science **245:** 1505–1509.
21. ISSA, A.M. *et al.* 1990. Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. J. Neurosci. **10:** 3247–3254.
22. BHATNAGAR, S., N. SHANKS & M.J. MEANEY. 1996. Plaque-forming cell responses and antibody titers following injection of sheep red blood cells in nonstressed, acute,

and/or chronically stressed handled and nonhandled animals. Dev. Psychobiol. **29:** 171–181.
23. LABAN, O. *et al.* 1995. Experimental allergic encephalomyelitis in adult DA rats subjected to neonatal handling or gentling. Brain Res. **676:** 133–140.
24. MASON, D. 1991. Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol. Today **12:** 57-60.
25. PLOTSKY, P.M. & M.J. MEANEY. 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res. Mol. Brain. Res. **18:** 195–200.
26. FRANCIS, D., P.M. PLOTSKY & M.J. MEANEY. 2004. Handling increases CRH mRNA expression in selected hypothalamic neuronal populations. Submitted for publication.
27. PAGE, M.E. & R.J. VALENTINO. 1994. Locus coeruleus activation by physiological challenges. Brain Res. Bull. **35:** 557–560.
28. PEARSON, D. *et al.* 1997.The effect of postnatal environment on stress-induced changes in hippocampal FOS-like immunoreactivity in adult rats. Soc. Neurosci. Abstr. **23:** 1849.
29. LIU, D. *et al.* 2000. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinephrine release in the hypothalamic paraventricular nucleus. J. Neuroendocrinol. **12:** 5–12.
30. DE KLOET, E.R. 2000. Stress in the brain. Eur. J. Pharmacol. **405:** 187–198.
31. MEANEY, M.J. *et al.* 1985. Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. Behav. Neurosci. **99:** 765–770.
32. SARRIEAU, A., S. SHARMA & M.J. MEANEY. 1988. Postnatal development and environmental regulation of hippocampal glucocorticoid and mineralocorticoid receptors. Brain Res. **471:** 158–162.
33. O'DONNELL, D. *et al.* 1994. Postnatal handling alters glucocorticoid, but not mineralocorticoid messenger RNA expression in the hippocampus of adult rats. Brain Res. Mol. Brain Res. **26:** 242–248.
34. JACOBSON, L. & R. SAPOLSKY. 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr. Rev. **12:** 118–134.
35. DIORIO, D., V. VIAU & M.J. MEANEY. 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J. Neurosci. **13:** 3839–3847.
36. DE KLOET, E.R. *et al.* 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev. **19:** 269–301.
37. OWENS, M.J., M.A. VARGAS & C.B. NEMEROFF. 1993. The effects of alprazolam on corticotropin-releasing factor neurons in the rat brain: implications for a role for CRF in the pathogenesis of anxiety disorders. J. Psychiatr. Res. **27:** 209–220.
38. DE BOER, S.F., J.L. KATZ & R.J. VALENTINO. 1992. Common mechanisms underlying the proconflict effects of corticotropin-releasing factor, a benzodiazepine inverse agonist and electric foot-shock. J. Pharmacol. Exp. Ther. **262:** 335–342.
39. CALDJI, C. *et al.* 2000. The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. Neuropsychopharmacology **22:** 219–229.
40. CALDJI, C. *et al.* 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. USA **95:** 5335–5340.
41. WILSON, M.A. 1996. GABA physiology: modulation by benzodiazepines and hormones. Crit. Rev. Neurobiol. **10:** 1–37.
42. MEHTA, A.K. & M.K. TICKU. 1999. An update on GABAA receptors. Brain Res. Brain Res. Rev. **29:** 196–217.
43. NUTT, D.J. *et al.* 1992. Clinical correlates of benzodiazepine receptor function. Clin. Neuropharmacol. **15:** 679A–680A.
44. HENNESSY, M.B., J. LI & S. LEVINE. 1980. Infant responsiveness to maternal cues in mice of 2 inbred lines. Dev. Psychobiol. **13:** 77-84.

45. LEE, M.H. & D.I. WILLIAMS. 1975. Long term changes in nest condition and pup grouping following handling of rat litters. Dev. Psychobiol. **8:** 91–95.
46. LIU, D. *et al.* 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science **277:** 1659–1662.
47. FRANCIS, D. *et al.* 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science **286:** 1155–1158.
48. STERN, J.M. 1997. Offspring-induced nurturance: animal-human parallels. Dev. Psychobiol. **31:**19–37.
49. CHAMPAGNE, F.A. *et al.* 2003. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol. Behav. **79:** 359–371.
50. PURVES, D., W.D. SNIDER & J.T. VOYVODIC. 1988. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. Nature **336:** 123–128.
51. LEVINE, S. & G.W. LEWIS. 1959. The relative importance of experimenter contact in an effect produced by extra-stimulation in infancy. J. Comp. Physiol. Psychol. **52:** 368–369.
52. MEANEY, M.J. & D.H. AITKEN. 1985. The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations: temporal parameters. Brain Res. **354:** 301–304.
53. MEANEY, M.J. *et al.* 1994. Corticosteroid receptors in rat brain and pituitary during development and hypothalamic-pituitary-adrenal (HPA) function. *In* Receptors and the Developing Nervous System. P.A.Z. McLaughlin, Ed.: 163–202. Chapman and Hall. London.
54. MEANEY, M.J., D.H. AITKEN & R.M. SAPOLSKY. 1987. Thyroid hormones influence the development of hippocampal glucocorticoid receptors in the rat: a mechanism for the effects of postnatal handling on the development of the adrenocortical stress response. Neuroendocrinology **45:** 278–283.
55. BANKER, G.A. & W.M. COWAN. 1977. Rat hippocampal neurons in dispersed cell culture. Brain Res. **126:** 397–342.
56. MITCHELL, J.B. *et al.* 1990. Serotonin regulates type II corticosteroid receptor binding in hippocampal cell cultures. J. Neurosci. **10:** 1745–1752.
57. SAVARD, P. *et al.* 1984. Effect of neonatal hypothyroidism on the serotonin system of the rat brain. Brain Res. **292:** 99–108.
58. MITCHELL, J.B., L.J. INY & M.J. MEANEY. 1990. The role of serotonin in the development and environmental regulation of type II corticosteroid receptor binding in rat hippocampus. Brain Res. Dev. Brain Res. **55:** 231–235.
59. SMYTHE, J.W., W.B. ROWE & M.J. MEANEY. 1994. Neonatal handling alters serotonin (5-HT) turnover and 5-HT2 receptor binding in selected brain regions: relationship to the handling effect on glucocorticoid receptor expression. Brain Res. Dev. Brain Res. **80:** 183–189.
60. MITCHELL, J.B. *et al.* 1992. Serotonergic regulation of type II corticosteroid receptor binding in hippocampal cell cultures: evidence for the importance of serotonin-induced changes in cAMP levels. Neuroscience **48:** 631–639.
61. SANDERS-BUSH, E., M. TSUTSUMI & K.D. BURRIS. 1990. Serotonin receptors and phosphatidylinositol turnover. Ann. N.Y. Acad. Sci. **600:** 224–235; discussion 235–236.
62. YOSHIMASA, T. *et al.* 1987. Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature **327:** 67–70.
63. PARENT, M. *et al.* 2001. Analysis of amino acids and catecholamines, 5-hydroxytryptamine and their metabolites in brain areas in the rat using in vivo microdialysis.Methods **23:** 11–20.
64. IKE, J., H. CANTON & E. SANDERS-BUSH. 1995. Developmental switch in the hippocampal serotonin receptor linked to phosphoinositide hydrolysis. Brain Res. **678:** 49–54.
65. LUCAS, J.J. & R. HEN. 1995. New players in the 5-HT receptor field: genes and knockouts. Trends Pharmacol. Sci. **16:** 246–252.
66. PEPIN, M.C., S. BEAULIEU & N. BARDEN. 1989. Antidepressants regulate glucocorticoid receptor messenger RNA concentrations in primary neuronal cultures. Brain Res. Mol. Brain Res. **6:** 77–83.
67. PEPIN, M.C., M.V. GOVINDAN & N. BARDEN. 1992. Increased glucocorticoid receptor gene promoter activity after antidepressant treatment. Mol. Pharmacol. **41:** 1016–1022.

68. LAPLANTE, P., J. DIORIO & M.J. MEANEY. 2002. Serotonin regulates hippocampal glucocorticoid receptor expression via a 5-HT7 receptor. Brain Res. Dev. Brain Res. **139:** 199–203.
69. FRANCIS, D. *et al.* 1996. The role of early environmental events in regulating neuroendocrine development. Moms, pups, stress, and glucocorticoid receptors. Ann. N.Y. Acad. Sci. **794:** 136–152.
70. MEANEY, M.J. *et al.* 2000. Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. J. Neurosci. **20:** 3926–3935.
71. DE GROOT, R.P. & P. SASSONE-CORSI. 1993. Hormonal control of gene expression: multiplicity and versatility of cyclic adenosine 3′ ,5′ -monophosphate-responsive nuclear regulators. Mol. Endocrinol. **7:** 145–153.
72. HABENER, J.F. 1990. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol. Endocrinol. **4:** 1087–1094.
73. IMAGAWA, M., R. CHIU & M. KARIN. 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51:** 251–260.
74. MONTMINY, M.R., G.A. GONZALEZ & K.K. YAMAMOTO. 1990. Regulation of cAMP-inducible genes by CREB. Trends Neurosci. **13:** 184–188.
75. SHENG, M. & M.E. GREENBERG. 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron **4:** 477-485.
76. VALLEJO, M. 1994. Transcriptional control of gene expression by cAMP-response element binding proteins. J. Neuroendocrinol. **6:** 587-596.
77. WALTON, K.M. & R.P. REHFUSS. 1990. Molecular mechanisms of cAMP-regulated gene expression. Mol. Neurobiol. **4:** 197–210.
78. YAMAMOTO, K.K. *et al.* 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature **334:** 494-498.
79. VACCARINO, F.M. *et al.* 1993. Induction of immediate early genes by cyclic AMP in primary cultures of neurons from rat cerebral cortex. Brain Res. Mol. Brain Res. **19:** 76-82.
80. LECLERC, S. *et al.* 1991. Purification of a human glucocorticoid receptor gene promoter-binding protein. Production of polyclonal antibodies against the purified factor. J. Biol. Chem. **266:** 8711-8719.
81. STRAHLE, U. *et al.* 1992. At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc. Natl. Acad. Sci. USA **89:** 6731-6735.
82. MCCORMICK, J.A. *et al.* 2000. 5′ -heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. Mol. Endocrinol. **14:** 506–517.
83. CHEN, F., C.S. WATSON & B. GAMETCHU. 1999. Multiple glucocorticoid receptor transcripts in membrane glucocorticoid receptor-enriched S-49 mouse lymphoma cells. J. Cell. Biochem. **74:** 418-429.
84. NOBUKUNI, Y. *et al.* 1995. Characterization of the human glucocorticoid receptor promoter. Biochemistry **34:** 8207-8214.
85. CROSBY, S.D. *et al.* 1991. The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol. Cell. Biol. **11:** 3835–3841.
86. RAZIN, A. 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. EMBO J. **17:** 4905-4908.
87. SZYF, M. 2001. Towards a pharmacology of DNA methylation. Trends Pharmacol. Sci. **22:** 350–354.
88. SAPIENZA, C. 1990. Parental imprinting of genes. Sci. Am. **263:** 52-60.
89. RAZIN, A. & H. CEDAR. 1977. Distribution of 5-methylcytosine in chromatin. Proc. Natl. Acad. Sci. USA **74:** 2725–2728.
90. MYERS, M.M. *et al.* 1989. Relationships between maternal behavior of SHR and WKY dams and adult blood pressures of cross-fostered F1 pups. Dev. Psychobiol. **22:** 55-67.
91. KESHET, I., J. YISRAELI & H. CEDAR. 1985. Effect of regional DNA methylation on gene expression. Proc. Natl. Acad. Sci. USA **82:** 2560–2564.
92. CLARK, S.J. *et al.* 1994. High sensitivity mapping of methylated cytosines. Nucleic Acids Res. **22:** 2990–2997.

93. FROMMER, M. *et al.* 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. USA **89:** 1827–1831.
94. HERSHKO, A.Y. *et al.* 2003. Methylation of HoxA5 and HoxB5 and its relevance to expression during mouse development. Gene **302:** 65–72.
95. MEANEY, M.J. *et al.* 1996. Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. Dev. Neurosci. **18:** 49–72.
96. NAN, X. *et al.* 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature **393:** 386–389.
97. COMB, M. & H.M. GOODMAN. 1990. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. **18:** 3975–3982.
98. MILBRANDT, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science **238:** 797–799.
99. WEAVER, I.C. *et al.* Epigenomic programming by maternal behavior. Nat. Neurosci.
100. AGRAWAL, A.A. 2001. Phenotypic plasticity in the interactions and evolution of species. Science **294:** 321–326.
101. ROSSITER, M.C. 1999. Maternal Effects as Adaptations. T.A.F. Mousseau, Ed. Oxford University Press. London.