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Epigenetic programming by maternal behavior

Ian C G Weaver[1,2], Nadia Cervoni[3], Frances A Champagne[1,2], Ana C D'Alessio[3], Shakti Sharma[1], Jonathan R Seckl[4], Sergiy Dymov[3], Moshe Szyf[2,3] & Michael J Meaney[1,2]

Here we report that increased pup licking and grooming (LG) and arched-back nursing (ABN) by rat mothers altered the offspring epigenome at a glucocorticoid receptor (GR) gene promoter in the hippocampus. Offspring of mothers that showed high levels of LG and ABN were found to have differences in DNA methylation, as compared to offspring of 'low-LG-ABN' mothers. These differences emerged over the first week of life, were reversed with cross-fostering, persisted into adulthood and were associated with altered histone acetylation and transcription factor (NGFI-A) binding to the GR promoter. Central infusion of a histone deacetylase inhibitor removed the group differences in histone acetylation, DNA methylation, NGFI-A binding, GR expression and hypothalamic-pituitary-adrenal (HPA) responses to stress, suggesting a causal relation among epigenomic state, GR expression and the maternal effect on stress responses in the offspring. Thus we show that an epigenomic state of a gene can be established through behavioral programming, and it is potentially reversible.

Through undefined epigenetic processes, maternal effects influence the development of defensive responses to threat in organisms ranging from plants to mammals[1,2]. In the rat, such effects are mediated by variations in maternal behavior, which serve as the basis for the transmission of individual differences in stress responses from mother to offspring[3–5]. Mother-pup contact in the rat primarily occurs within the context of a nest-bout, which begins when the mother approaches the litter, licks and grooms her pups, and nurses while occasionally licking and grooming the pups[6]. There are stable individual differences in two forms of maternal behavior—LG and ABN—over the first week of lactation[6–10]. Such naturally occurring variations in maternal behavior are associated with the development of individual differences in behavioral and HPA responses to stress in the offspring. As adults, the offspring of 'high-LG-ABN' mothers are less fearful and show more modest HPA responses to stress than the offspring of 'low-LG-ABN' mothers[6–9]. Cross-fostering studies show that the biological offspring of low-LG-ABN mothers reared by high-LG-ABN dams resemble the normal offspring of high-LG-ABN mothers (and vice versa[9]). These findings suggest that variations in maternal behavior serve as a mechanism for the nongenomic transmission of individual differences in stress reactivity across generations[4,5,9]. The critical question concerns the mechanisms whereby these maternal effects, or other forms of environmental 'programming', are sustained over the lifespan of the animal.

Maternal behavior in the rat permanently alters the development of HPA responses to stress through tissue-specific effects on gene expression. The magnitude of the HPA response to acute stress is a function of hypothalamic corticotropin-releasing factor (CRF) release, which activates the pituitary-adrenal system. There are also modulatory influences, such as glucocorticoid negative feedback that inhibits CRF synthesis and release, thus dampening HPA responses to stress[11]. The adult offspring of high- compared with low-LG-ABN mothers show increased hippocampal GR expression and enhanced glucocorticoid feedback sensitivity[7,9]. Predictably, adult offspring of high-LG-ABN mothers show decreased hypothalamic CRF expression and more modest HPA responses to stress[7]. Eliminating the difference in hippocampal GR levels abolishes the effects of early experience on HPA responses to stress in adulthood[12], suggesting that the difference in hippocampal GR expression serves as a mechanism for the effect of early experience on the development of individual differences in HPA responses to stress[5].

In vivo and *in vitro* studies suggest that maternal LG and ABN increase GR gene expression in the offspring through increased serotonin (5-HT) activity at 5-HT$_7$ receptors, and the subsequent activation of cAMP and cAMP-dependent protein kinase activity[13–15]. Both the *in vitro* effect of 5-HT and the *in vivo* effect of maternal behavior on GR gene expression are accompanied by an increased hippocampal expression of nerve growth factor-inducible protein A (NGFI-A, a transcription factor also known as egr-1, krox-24, zenk and zif-268). The non-coding exon 1 region of the hippocampal GR includes a promoter region, exon 1$_7$, containing a binding site for NGFI-A[16] (**Fig. 1a**). Splice variants of the GR mRNA containing the exon 1$_7$ sequence are found predominantly in the brain, and the expression of GR mRNAs containing the exon 1$_7$ sequence is increased in the offspring of high-LG-ABN mothers or following manipulations that increase maternal licking and grooming[16] (Weaver, I.C.G. *et al.*, *Soc. Neurosci. Abstr.* 697.15, 2001), suggesting that the use of this promoter is enhanced as a function of maternal care. Although these findings might explain the increased GR expression in the neonate, we are left with the question of how the effect of maternal care might persist into

[1]Douglas Hospital Research Center, 6875 LaSalle Blvd., Montréal, Québec H4H 1R3, Canada. [2]McGill Program for the Study of Behaviour, Genes and Environment and [3]Department of Pharmacology and Therapeutics, McGill University, 3655 Sir William Osler Promenade, Montréal, Québec H3G 1Y6, Canada. [4]Molecular Medicine Centre, Edinburgh University, Western General Hospital, Edinburgh EH4 2XU, UK. Correspondence should be addressed to M.J.M. (michael.meaney@mcgill.ca) or M.S. (moshe.szyf@mcgill.ca).

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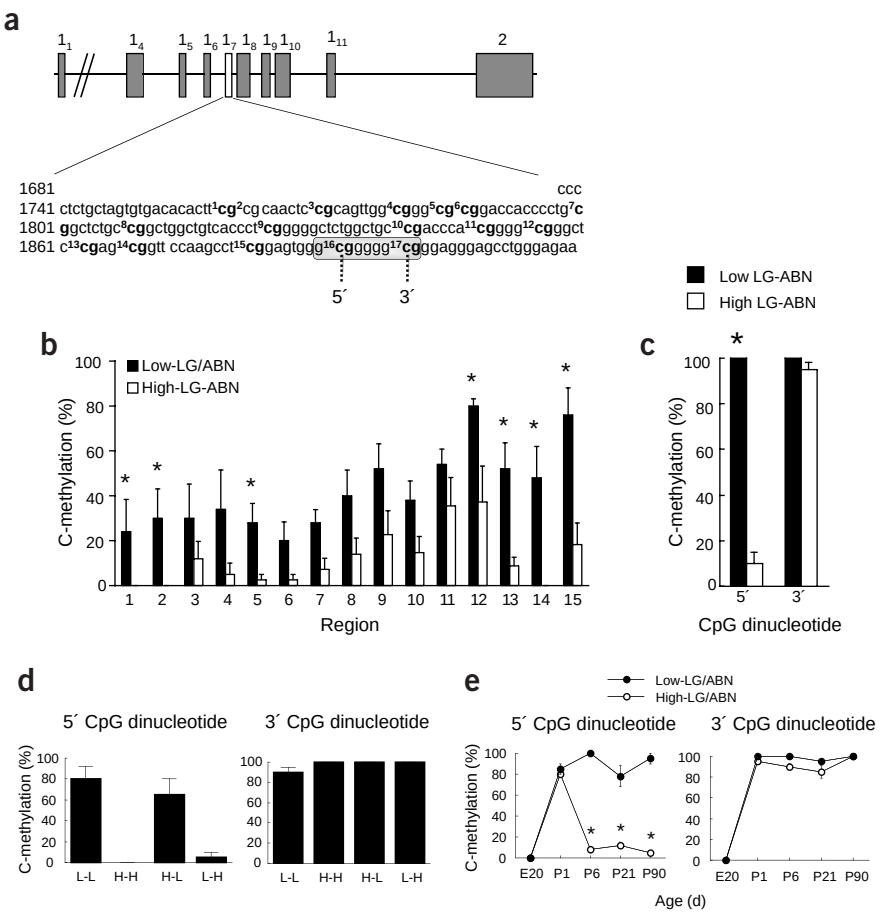

Figure 1 Maternal care alters cytosine methylation of GR promoter. (**a**) Sequence map of the exon 1_7 GR promoter including the 17 CpG dinucleotides (bold) and the NGFI-A binding region[16] (encircled). (**b**,**c**) Methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter region from adult high- and low-LG-ABN offspring (6–10 clones sequenced/animal; $n = 4$ animals/group; $^*P < 0.01$). (**b**) Percentage of cytosine residues that were methylated (mean ± s.e.m.) for the first 15 CpG dinucleotides ($^*P < 0.05$). (**c**) Percentage of methylated cytosines (mean ± s.e.m.) for the 5′ (site 16) and 3′ (site 17) CpG dinucleotides within the NGFI-A binding sequence ($^*P < 0.0001$). (**d**) The effect of cross-fostering the offspring of high- and low-LG-ABN mothers on cytosine methylation of the 5′ and 3′ CpG dinucleotides within the NGFI-A binding sequence of the exon 1_7 GR promoter gene in adult hippocampi ($n = 5$ animals/group). L-L: animals born to and reared by low-LG-ABN mothers; H-H: animals born to and reared by high-LG-ABN mothers; H-L: animals born to high-LG-ABN mothers and reared by low-LG-ABN mothers; L-H: animals born to low-LG-ABN mothers and reared by high-LG-ABN mothers. (**e**) Percentage of cytosine methylation (mean ± s.e.m.) of the 5′ and 3′ CpG dinucleotides within the NGFI-A binding region of the exon 1_7 GR promoter gene in the offspring of high- or low-LG-ABN mothers ($n = 5$ animals/group; $P < 0.001$) as a function of age. There were no differences at any postnatal age in level of cytosine methylation of the 3′ CpG (site 17).

adulthood. Gene expression is controlled by the epigenome, which is comprised of chromatin structure[17] and DNA methylation[18]. We tested the hypothesis that maternal care alters DNA methylation of the GR exon 1_7 promoter, and that these changes are stably maintained into adulthood and associated with differences in GR expression and HPA responses to stress.

RESULTS

Maternal care and methylation of exon 1_7 promoter

DNA methylation is a stable, epigenomic mark at CpG dinucleotides often associated with stable variations in gene transcription[18–20]. Two kinds of changes in DNA methylation are known to affect gene expression: regional, non-site specific DNA methylation around a promoter[19] and site-specific methylation. Hypomethylation of CpG dinucleotides of regulatory regions of genes is associated with active chromatin structure and transcriptional activity[18,20]. 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 GR promoter, which is activated in the hippocampus in offspring of high-LG-ABN mothers.

To determine whether DNA methylation of specific target sites on the GR promoter change in response to maternal care, we mapped differences in the methylation status of individual cytosines within the CpG dinucleotides of the exon 1_7 promoter from hippocampal tissue from the adult offspring of high- and low-LG-ABN mothers. We used sodium bisulfite mapping[21,22], with a particular interest in the region around the NGFI-A consensus sequence (**Fig. 1a**). The results showed significant differences in the methylation of specific sites of the exon 1_7 GR promoter sequence (**Fig. 1b**,**c**). A two-way ANOVA revealed a highly significant effect of Group ($F = 55.9$, $P < 0.0001$) and Region ($F = 27.7$, $P < 0.0001$), as well as a significant Group × Region interaction effect ($F = 27.7$, $P < 0.0001$). Importantly, the cytosine residue within the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence (**Fig. 1c**) is always methylated in the offspring of low-LG-ABN mothers, and rarely methylated in the offspring of high-LG-ABN dams. In contrast, the 3′ CpG dinucleotide (site 17) remains methylated, regardless of differences in maternal care. Dissected hippocampi inevitably contain glial cells as well as neurons. Considering the pronounced effect of maternal care on the methylation status of the 5′ CpG dinucleotide of the NGFI-A response element (>90%), the effect of maternal care must include neuronal as well as glial cells; both populations express GR[23,24] and NGFI-A[25] genes.

Cross-fostering reveals epigenetic marking by maternal behavior

Our findings suggest that specific sites within the exon 1_7 GR promoter are differentially methylated as a function of maternal behavior, but these findings are merely correlational. To directly examine the relation between maternal behavior and DNA methylation within the exon 1_7 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-LG-ABN dams within 12 h of birth[9]. Cross-fostering produced a pattern of exon 1_7 promoter methylation that was associated with the rearing mother ($F = 4.8$, $P < 0.05$; **Fig. 1d**) and thus reversed the difference in methylation at specific cytosines, notably at the 5′ CpG dinucleotide (site 16) of the

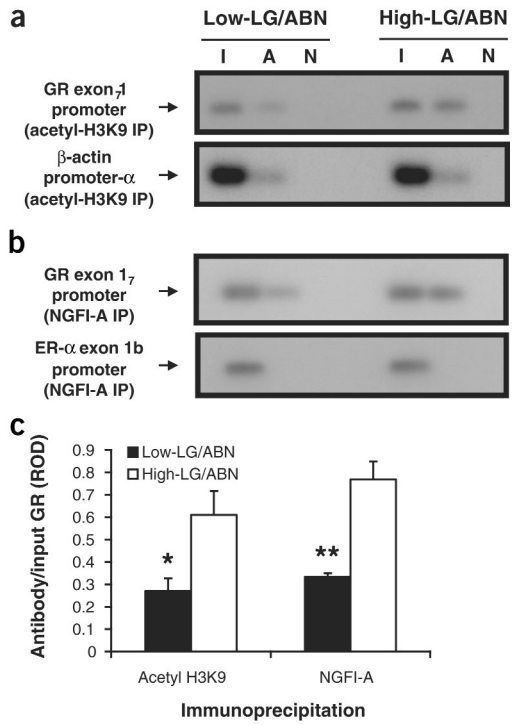

Figure 2 Chromatin immunoprecipitation analysis of the association between histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR sequence in hippocampal tissue from adult offspring of high- and low-LG-ABN mothers ($n = 4$ animals/group). (**a**,**b**) Lanes were loaded with non-immunoprecipitated input (I), acetylated histone H3-K9 (top) or NGFI-A (middle) primary antibody immunoprecipitated (A), or non-immune IgG antibody immuno-precipitated (N) hippocampal extracts). (**a**) Representative Southern blot of the amplified exon 1_7 region from acetyl-histone H3-K9 immunoprecipitated hippocampal tissue (194 bp band) and β-actin (171 bp band) control. (**b**) Representative Southern blot of the amplified exon 1_7 region of the GR from NGFI-A immunoprecipitated hippocampal tissue (194 bp band). DNA loading was controlled using primers specific for the ubiquitously expressed β-actin promoter-α region. Exon 1b estrogen receptor-α promoter region, which does not contain NGFI-A recognition elements (493 bp), amplified from the same NGFI-A immunoprecipitated hippocampal tissue was run as a control for specificity and showed no signal. (**c**) Relative optical density (ROD; mean ± s.e.m.) of exon 1_7 sequence amplified from acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue of adult high- and low-LG-ABN offspring ($n = 4$ animals/group; $^*P < 0.001$; $^{**}P < 0.0001$).

NGFI-A consensus sequence (**Fig. 1d**, left panel). Thus, in the low-LG-ABN offspring that were fostered to high-LG-ABN dams, methylation of this 5′ site within the exon 1_7 promoter was indistinguishable from that of the biological offspring of high-LG-ABN mothers. Likewise, the methylation of the same 5′ CpG dinucleotide in the biological offspring of high-LG-ABN mothers reared by low-LG-ABN dams was comparable to that of low-LG-ABN offspring. There was no effect of cross-fostering at the cytosine within the 3′ CpG dinucleotide (site 17; **Fig. 1d**).

These findings suggest that variations in maternal care directly alter the methylation status of the exon 1_7 promoter of the GR gene. Thus we have demonstrated that a DNA methylation pattern can be established through a behavioral mode of programming without germ line transmission. In parental imprinting, a well-established paradigm of inheritance of an epigenomic mark, the paternally and maternally inherited alleles are differentially methylated. These methylation patterns are defined during maturation of spermatocytes and oocytes, and are transmitted to the offspring through the germ line[26].

Timing of the maternal effect on DNA methylation

The maternal care of high- and low-LG-ABN mothers differs only during the first week of life[7,8]. Thus, we wondered whether this period corresponds to the timing for the appearance of the difference in DNA methylation in the offspring. We used sodium bisulfite mapping to examine the methylation status of the cytosines within the exon 1_7 GR promoter during development (**Fig. 1e**). Statistical analysis of the data for the 5′ CpG (site 16) revealed a highly significant effect of Group ($F = 66.7$, $P < 0.0001$) and Age ($F = 21.1$, $P < 0.0001$) as well as a significant interaction effect ($F = 13.7$, $P < 0.0001$). Tukey *post-hoc* analysis revealed that the Group effect on methylation status of the 5′ CpG (site 16) was significant at P6, P21 and P90 ($P < 0.001$), but not at E20 or P1. Just before birth (embryonic day 20; E20) the entire region was unmethylated in both groups. Strikingly, one day after birth (postnatal day 1; P1) the exon 1_7 GR promoter was *de novo* methylated in both groups. The 5′ and 3′ CpG sites of the exon 1_7 GR NGFI-A response element in the offspring of both high- and low-LG-ABN mothers, which exhibit differential methylation later in life, were *de novo* methylated to the same extent. These data show that

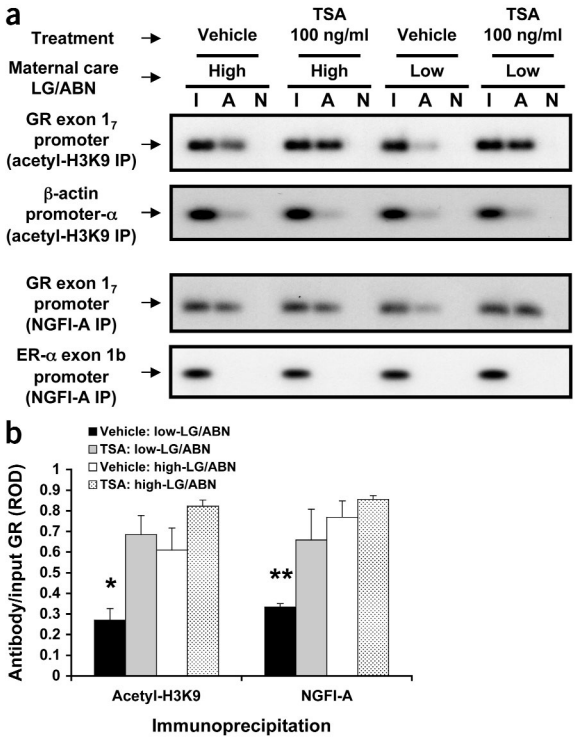

Figure 3 HDAC inhibitior (TSA) eliminates maternal effect on histone acetylation and NGFI-A binding. (**a**) Chromatin immunoprecipitation analysis of the association between histone H3-K9 acetylation and NGFI-A binding to the exon 1_7 GR promoter sequence in hippocampal tissue from vehicle- and TSA-treated (100 ng/ml) adult offspring of high- and low-LG-ABN mothers ($n = 4$ animals/group; lane labels as described in **Fig. 2**). (**b**) Relative optical density (ROD; mean ± s.e.m.) of exon 1_7 sequence amplified from acetyl-histone H3-K9 or NGFI-A immunoprecipitated hippocampal tissue ($^*P < 0.05$; $^{**}P < 0.01$).

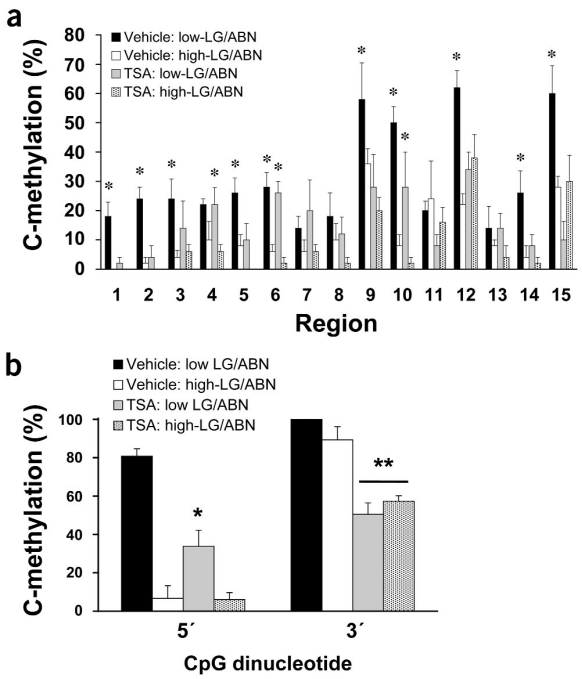

Figure 4 TSA effects on cytosine methylation. (**a**,**b**) Methylation analysis of the 17 CpG dinucleotides of the exon 1_7 GR promoter in hippocampi of vehicle- and TSA-treated (100 ng/ml) adult offspring of high- and low-LG-ABN mothers ($n = 5$ animals/group). (**a**) Percentage of cytosine residues that were methylated (mean ± s.e.m.) for the first 15 CpG dinucleotides ($^*P < 0.05$). (**b**) Percentage of methylated cytosines for the 5′ (site 16) and 3′ (site 17) CpG dinucleotides within the NGFI-A binding region ($^*P < 0.001$; $^{**}P < 0.003$).

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[27]. Because similar analyses are not documented for other genes, it remains unknown whether changes in methylation are common around birth or whether they are unique to this GR promoter.

The differences in the methylation status of the exon 1_7 GR promoter between the two groups developed between P1 and P6, the period when differences in the maternal behavior of high- and low-LG-ABN dams are apparent[5,8]. By P6, the NGFI-A response element 5′ CpG dinucleotide (site 16) was effectively 'demethylated' in the high-, but not in the low-LG-ABN group. The group difference in CpG dinucleotide methylation remains consistent through to adulthood (P90; **Fig. 1e**). These findings, together with those of the cross-fostering study, 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 indicate that the first week of postnatal life is a 'critical period' for the effects of early experience on hippocampal GR expression[28].

Maternal effects on chromatin structure and NGFI-A binding

The next question concerns the functional importance of such differences in methylation. DNA methylation is associated with changes in chromatin activity states[18]. Chromatin gates the accessibility of promoters to transcription factors[17]. Histone acetylation at the lysine-9 (K9) residue of H3 and H4 histones is a well-established marker of active chromatin[17,29]. Acetylation of the histone tails neutralizes the positively charged histones, which disrupts histone binding to negatively charged DNA and thus promotes transcription factor binding. We tested the hypothesis that the maternal effect on DNA methylation results in (i) increased histone acetylation at the K9 residue of the H3 histone(s) associated with the exon 1_7 GR promoter and (ii) increased interaction between NGFI-A and the promoter sequence. We performed a chromatin immunoprecipitation (ChIP) analysis of histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter in the native chromatin environment *in vivo*. Intact hippocampi from adult offspring of high- and low-LG-ABN mothers were crosslinked *in vivo* by paraformaldehyde perfusion. We then selectively immunoprecipitated protein-DNA complexes with either an acetylated H3-K9 histone primary antibody or an NGFI-A primary antibody. The protein-DNA complexes were uncrosslinked, and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the exon 1_7 GR promoter sequence. There were significant Group effects for the association of both histone H3-K9 acetylation ($t = 2.1$, $^*P < 0.001$) and NGFI-A ($t = 3.1$, $^{**}P < 0.0001$) with the exon 1_7 GR promoter sequence. These results indicated significantly greater histone H3-K9 acetylation association and threefold greater binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the adult offspring of high- compared with low-LG-ABN mothers (**Fig. 2**). Thus, maternal programming of the exon 1_7 GR promoter involves DNA methylation, histone H3-K9 acetylation and alterations in NGFI-A binding.

Reversal of maternally mediated epigenetic marking

These findings suggest that maternal care influences hippocampal GR expression, and thus HPA function in the offspring, through epigenetic alterations that regulate NGFI-A binding to the exon 1_7 promoter. A critical question is whether the impact of early experience is reversible and whether epigenetic programming is modifiable in adult, post-mitotic tissues? The generally accepted model is that the DNA methylation pattern is an irreversible reaction in adult post-mitotic cells. However, recent data from *in vitro* experiments suggests that in certain instances it is possible to induce replication-independent demethylation of ectopically methylated genes by increasing histone acetylation using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA)[29,30]. Cytosine methylation attracts methylated DNA binding proteins and HDACs that prevent histone acetylation and thus transcription factor binding[29,30]. Activation of chromatin through HDAC inhibition might trigger DNA demethylation by increasing the accessibility of DNA to demethylase activity[30]. We tested the hypothesis that inhibition of HDACs with TSA would result in increased K9 acetylation of H3-histones associated with the exon 1_7 GR promoter, DNA demethylation, NGFI-A binding and reversal of maternal programming of stress responses in the adult offspring of low-LG-ABN mothers.

We first used ChIP analysis to determine whether histone H3-K9 acetylation and NGFI-A protein binding to the exon 1_7 GR promoter is altered in the offspring of high- and low-LG-ABN mothers through intracerebroventricular (i.c.v.) infusion of the adult offspring with TSA (100 ng/ml) or vehicle. Statistical analysis revealed a significant Group × Treatment interaction effect for both the histone H3-K9 acetylation ($F = 4.93$, $P < 0.05$) and NGFI-A ($F = 8.97$, $P = 0.01$). *Post-hoc* analysis showed that for both assays, vehicle - treated offspring of low-LG-ABN mothers showed significantly ($^*P < 0.01$) less association than any other group. These results indicate greater histone H3-K9 acetylation association and more bind-

ing (>3 fold) of NGFI-A protein to the hippocampal exon 1_7 GR promoter in the adult offspring of TSA-treated low-LG-ABN mothers compared with the vehicle-treated offspring of low-LG-ABN mothers (**Fig. 3**); there were no significant differences between TSA-treated offspring of low-LG-ABN mothers and either TSA- or vehicle-treated offspring of high-LG-ABN dams. As expected, TSA treatment did not change histone H3-K9 acetylation or NGFI-A binding in the adult offspring of high-LG-ABN mothers, because the GR exon 1_7 promoter region in the offspring of high-LG-ABN mothers is normally associated with acetylated histones and highly bound with NGFI-A.

To determine whether TSA treatment reverses the maternal effect on methylation within specific CpG dinucleotides on the exon 1_7 GR promoter, we mapped the differences in methylation using the sodium bisulfite technique, focusing on the NGFI-A consensus sequence within the exon 1_7 region (**Fig. 1a**). Statistical analysis of the data across all 17 sites revealed a significant effect of Group ($F = 93.2$, $P < 0.0001$), Treatment ($F = 52.8$, $P < 0.0001$) and Region ($F = 30.4$, $P < 0.0001$), as well as a significant Group × Treatment × Region interaction ($F = 2.1$, $P = 0.01$), Group × Treatment interaction ($F = 19.9$, $P < 0.0001$), Group × Region interaction ($F = 4.1$, $P < 0.0001$) and Treatment × Region interaction ($F = 2.8$, $P < 0.0001$). The results again revealed significant differences in the methylation of a number of regions of the exon 1_7 GR promoter sequence (**Fig. 4**) with significant differences within the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides of the NGFI-A consensus sequence (**Fig. 4b**). Statistical analysis of the data from these two sites revealed a highly significant effect of Group ($F = 43.8$, $P < 0.0001$), Treatment ($F = 65.3$, $P < 0.0001$) and Region ($F = 113.3$, $P < 0.0001$), as well as a significant Group × Treatment interaction ($F = 16.0$, $P < 0.0001$), Group × Region interaction ($F = 37.8$, $P < 0.0001$) and Treatment × Region interaction ($F = 4.5$, $P = 0.04$). *Post-hoc* analysis revealed that TSA treatment significantly decreased the degree of cytosine methylation within the 5′ (site 16) CpG dinucleotide of the NGFI-A binding region of the exon 1_7 GR promoter in the offspring of low-LG-ABN mothers in comparison to vehicle-treated low-LG-ABN mothers (*$P < 0.001$).

TSA treatment produced 'demethylation' of the 5′ CpG (site 16) and 3′ CpG (site 17) dinucleotides in the offspring of low-LG-ABN mothers, and hypomethylation of the 3′ CpG (site 17) dinucleotide in the offspring of high-LG-ABN mothers (**Fig. 4b**). These findings suggest that TSA treatment can reverse the hypermethylated status of the exon 1_7 GR promoter in the offspring of low-LG-ABN mothers. TSA treatment resulted in a more extensive change in DNA methylation than maternal care *per se*, since the 3′ CpG (site 17) dinucleotide, which is unaffected by maternal behavior, is partially 'demethylated' in response to TSA treatment in both cohorts (**Fig. 4b**). Also, as in the original study (**Fig. 1b**), maternal care altered the methylation status of other CpG dinucleotides in the exon 1_7 sequence; in the case of sites 1, 2, 5, 12, 14 and 15, these effects were similarly reversed with central TSA infusion. The significance of these sites for transcription factor binding is currently unknown and thus a focus of ongoing studies. Thus, stable DNA methylation marking by maternal behavior is reversible in the adult offspring hippocampus by pharmacological modulation of chromatin structure. While TSA altered the methylation of the both the 5′ and 3′ CpG sites within the NGFI-A response element, the former appears to be critical for the effect on NGFI-A binding to the exon 1_7 promoter. In a previous *in vitro* study using electrophilic mobility shift assays (EMSA) with purified recombinant NGFI-A protein[31] and differentially methylated oligonucleotide sequences containing the NGFI-A response element, we found that methylation of the cytosine within the 5′ CpG dinucleotide (site 16) completely eliminated the binding of NGFI-A, whereas methylation of the cytosine within the 3′ CpG dinucleotide (site 17) only slightly reduced NGFI-A protein binding (I.C.G.W., M.S. & M.J.M., unpublished data).

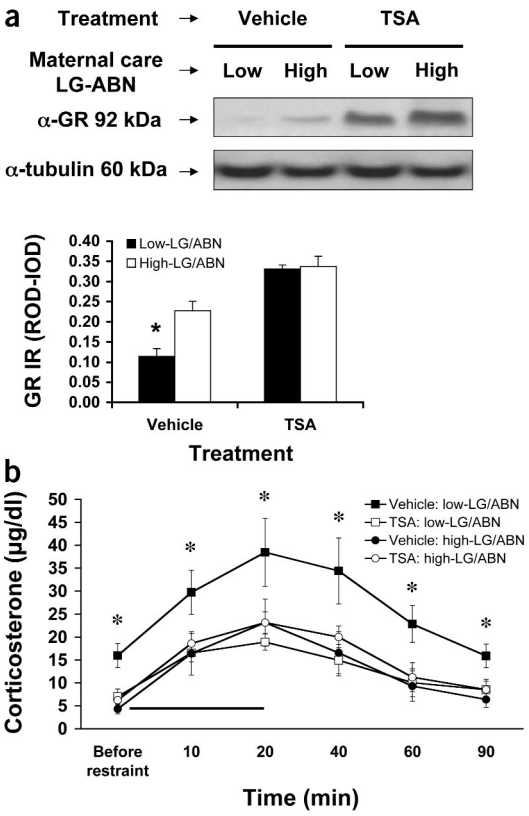

Figure 5 TSA eliminates the maternal effect on hippocampal GR expression and HPA responses to stress. (**a**) Top: a representative western blot showing absolute levels of electrophoresed hippocampal GR immunoreactivity (IR) from vehicle- and TSA (100 ng/ml)-treated adult offspring of high- or low-LG-ABN mothers. Molecular weight markers (SeeBlue, Santa Cruz Biotech) correspond to a single major band at 92 kDa. The middle panel shows the membrane reprobed for α-tubulin IR, illustrating absolute levels of electrophoresed hippocampal protein bound to the transfer membrane. Molecular weight markers correspond to a single major band at ~60 kDa and the intensity of the signal was similar in all lanes. The lower panel shows quantitative densitometric analysis (relative optical density, ROD) of GR IR levels from samples ($n = 5$ animals/group; *$P < 0.001$). (**b**) Plasma corticosterone responses[7] (mean ± s.e.m.) to a 20-min period of restraint stress (solid bar) in vehicle- and TSA (100 ng/ml)-treated adult offspring of high- or low-LG-ABN mothers ($n = 10$ animals/group; *$P < 0.01$).

Reversal of maternal effect on GR expression

GR gene expression in the hippocampus is increased in the adult offspring of high- compared with low-LG-ABN mothers[7,9]. We suggest that such differences are mediated by the differential methylation of the 5′ CpG dinucleotide (site 16) of the NGFI-A consensus sequence in the exon 1_7 GR promoter and the subsequent alteration of histone acetylation and NGFI-A binding to the exon 1_7 sequence. If the differential epigenetic marking regulates the expression of the exon 1_7 GR promoter in high- versus low-LG offspring, then reversal of the epigenetic marking should be accom-

panied by an increase in hippocampal GR expression. This hypothesis is supported by the results (**Fig. 5a**) showing that hippocampal GR expression was significantly increased in TSA-treated offspring of low-LG-ABN mothers to levels that were comparable to those of either the vehicle- or TSA-treated offspring of high-LG-ABN mothers. ANOVA revealed highly significant main effects of Group ($F = 7.4$, $P = 0.01$) and Dose ($F = 24.8$, $P < 0.0001$), as well as a significant Group × Dose interaction effect ($F = 3.1$, $P = 0.048$). *Post-hoc* analysis indicated that 100 ng/ml TSA treatment significantly increases hippocampal GR expression in the offspring of low-LG-ABN mothers (vehicle-treated low-LG-ABN vs. 100 ng/ml TSA-treated low-LG-ABN, $^*P < 0.001$), such that there is no longer a significant difference in hippocampal GR expression between the offspring of low- or high-LG-ABN mothers (100 ng/ml TSA treated low-LG-ABN vs. 100 ng/ml TSA treated high-LG-ABN, $P > 0.90$). Although TSA treatment significantly induced GR expression in low-LG-ABN adult offspring, global abundance of protein in the hippocampus was not apparently increased, as indicated by the equal α-tubulin immunoreactivity (**Fig. 5a**). In comparing the vehicle-treated groups, note that the effect of maternal care on GR gene expression[7,9] is subtler than the more pronounced effect on the methylation status of the 5′ CpG dinucleotide (site 16) within the exon 1_7 promoter (**Fig. 4b**). However, in previous studies[16] we found evidence for multiple promoters regulating hippocampal GR expression suggesting that exon 1_7 is but one of the regulatory sequences determining GR expression within the hippocampus.

Reversal of maternal effect on HPA responses to stress

As adults, the offspring of high-LG-ABN mothers show more modest HPA responses to stress than the offspring of low-LG-ABN mothers[7]. The effect of maternal care on HPA responses to stress seems to be, in part, associated with differences in hippocampal GR levels and glucocorticoid negative feedback sensitivity[7]. Given that TSA treatment reversed the group difference in hippocampal GR expression, we examined the adrenocortical responses to stress in a separate cohort of vehicle- and TSA-treated animals. Central infusion of TSA completely eliminated the maternal effect on HPA responses to acute stress (**Fig. 5b**). Statistical analysis of the plasma corticosterone data revealed significant effects of Group ($F = 4.3$, $P = 0.048$), Treatment ($F = 4.3$, $P = 0.046$) and Time ($F = 27.3$, $P < 0.0001$), as well as a significant Group × Treatment interaction effect ($F = 7.7$, $P = 0.009$). *Post-hoc* analysis revealed that TSA treatment significantly decreased basal plasma corticosterone in the offspring of low-LG-ABN mothers to levels comparable to those of high-LG-ABN animals. Thus, plasma corticosterone responses to restraint stress in the vehicle-treated adult offspring of low-LG-ABN mothers were significantly ($P < 0.01$) greater than those of TSA- and vehicle-treated adult offspring of high-LG-ABN mothers or TSA-treated offspring of Low-LG-ABN mothers. The HPA response to stress in the offspring of high-LG-ABN mothers was unaffected by TSA treatment.

In summary, central infusion of the HDAC inhibitor TSA enhanced histone H3-K9 acetylation of the exon 1_7 GR promoter in the offspring of the low-LG-ABN mothers, increased NGFI-A binding to its cognate sequence, induced hypomethylation of CpG dinucleotide sequences in the promoter and eliminated the maternal effect on hippocampal GR expression and the HPA response to stress.

These findings are consistent with idea that the maternal effect on GR expression and HPA responses to stress is mediated by alterations in chromatin structure. We propose that the reduced binding of NGFI-A to its response element on the hypoacetylated and hypermethylated exon 1_7 GR promoter contributes to the attenuation of GR expression in low-LG-ABN adult offspring, whereas increased NGFI-A binding to the hyperacetylated and hypomethylated response element on the exon 1_7 GR promoter in the offspring of the high-LG-ABN mothers would serve to maintain the differences in gene expression. DNA methylation represents a stable epigenetic mark; therefore, our findings provide an explanation for the enduring effect on mother-infant interactions over the first week of postnatal life on HPA responses to stress in the offspring.

DISCUSSION

Further studies are required to determine how maternal behavior alters the epigenetic status of the exon 1_7 GR promoter. In addition, the exact causal relationship between DNA methylation and altered histone acetylation and NGFI-A binding remains to be defined. Nevertheless, our findings provide the first evidence that maternal behavior produces stable alterations of DNA methylation and chromatin structure, providing a mechanism for the long-term effects of maternal care on gene expression in the offspring. 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 lifespan. 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[1,2] 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 whereby the experience of the mother is translated through an epigenetic mechanism of inheritance into phenotypic variation in the offspring. Thus, maternal effects could result in the transmission of adaptive responses across generations[1,2,5]. Indeed, among mammals, natural selection may have shaped offspring to respond to subtle variations in parental behavior as a forecast of the environmental conditions they will ultimately face once they become independent of the parent[5]. Epigenetic modifications of targeted regulatory sequences in response to even reasonably subtle variations in environmental conditions might then 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 effects on chromatin structure such as those described here serve as an intermediate process that imprints dynamic environmental experiences on the fixed genome, resulting in stable alterations in phenotype.

METHODS

Animals and maternal behavior. The animals used in all studies were derived from Long-Evans hooded rats born in our colony from animals originally obtained from Charles River Canada (St. Constant, Québec). All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocol approved by the McGill University Animal Care Committee. Maternal behavior was scored[32] and the adoption study was performed[9] as previously described. For further methodological details, see **Supplementary Methods** online.

Sodium bisulfite mapping. Sodium bisulfite mapping was performed as previously described[21,22]. The rat GR exon 1_7 genomic region (GenBank accession number AJ271870) of the sodium bisulfite-treated hippocampal DNA (50 ng/ml) was subjected to PCR amplification using outside primers (forward: 1646-TTTTTTAGGTTTTTTTAGAGGG-1667; reverse: 1930-

ATTTCTTTAATTTCTCTTCTCC-1908). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (2 min 30 s, 56 °C) and extension (1 min, 72 °C), followed by a final extension cycle (5 min, 72 °C) terminating at 4 °C. The PCR product (285 bp) was used as a template for subsequent PCR reactions using nested primers (forward: 1738-TTTTTTTGTTAGTGT-GATATATTT-1761; reverse: 1914-TTCTCCCAAACTCCCTCC-1897). The nested PCR product (177 bp) was then subcloned (Original TA cloning kit, Invitrogen), transformed, and ten different clones per plate were mini-prepped. Ten plasmids containing the ligated exon 1_7 GR promoter DNA fragment were sequenced per animal (T7 sequencing kit, USB, Amersham Pharmacia Biotech) starting from procedure C in the manufacturer's protocol. The sequencing reactions were resolved on a denaturing (6%) PAGE and visualized by autoradiography.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays[33] were done using the ChIP assay kit protocol (06-599, Upstate Biotechnology). Hippocampi were dissected from each rat brain and chromatin was immunoprecipitated using one of the following: rabbit polyclonal antibody to acetyl-histone H3 (Upstate Cell Signaling Solutions), rabbit polyclonal antibody to NGFI-A or normal rabbit IgG non-immune antibody (both from Santa Cruz Biotechnology). One-tenth of the lysate was kept to quantify the amount of DNA present in different samples before immunoprecipitation (input). The rat hippocampal GR exon 1_7 promoter region (GenBank accession number AJ271870) of the uncrosslinked DNA was subjected to PCR amplification (forward primer: 1750-TGTGACACACTTCGCGCA-1767; reverse primer: 1943-GGAGGGAAACCGAGTTTC-1926). PCR reactions were done with the FailSafe PCR system protocol using FailSafe PCR 2× PreMix D (Epicentre, InterScience). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (1 min, 56 °C) and extension (1 min, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. To control for unequal loading of acetyl-histone H3-K9 immunoprecipitate, the rat hippocampal β-actin promoter-α region (GenBank accession number V01217) of the uncrosslinked DNA was subjected to PCR amplification (forward primer: 10-TCAACTCACTTC TCTCTACT-29; reverse primer: 161-GCAAGGCTTTAACGGAAAAT-180). PCR reactions were done with the same protocol, but using FailSafe PreMix L (Epicentre, InterScience) with the same thermocycler protocol as previously described. To control for purity of the NGFI-A immunoprecipitate, we PCR-amplified the rat hippocampal exon 1b estrogen receptor-α promoter region (GenBank accession number X98236) of the uncrosslinked DNA (forward primer: 1836-GAAGAAACTCCCCTCAGCAT-1855; reverse primer: 2346-GAAATCAAAACACCGATCCT-2327), this time using FailSafe PreMix A (Epicentre, InterScience). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 34 cycles of denaturation (1 min, 95 °C), annealing (1 min, 60 °C) and extension (1 min, 72 °C), followed by a final extension cycle (10 min, 72 °C) terminating at 4 °C. PCR reactions on DNA purified from non-immunoprecipitated samples and immunoprecipitated samples were repeated exhaustively using varying amounts of template to ensure that results were within the linear range of the PCR. Products were separated on a 2% agarose gel to visualize bands corresponding to the exon 1_7 GR promoter (194 bp), β-actin promoter-α (171 bp) or exon 1b estrogen receptor-α promoter (493 bp) DNA fragments. Nucleic acids were transferred by Southern blot (overnight, 22 °C) to positively charged nylon transfer membrane (Hybond-N+, Amersham). An oligonucleotide (20 bp) specific for the exon 1_7 GR promoter sequence (GenBank accession number AJ271870; forward: 1881-TCCCGAGCGGTTCCAAGCCT-1907) was synthesized, as well as an oligonucleotide (21 bp) specific for the β-actin promoter-α sequence (GenBank accession number V01217; forward: 95-GTAAAAAAATGCTG-CACTGTC-115) and an oligonucleotide (20 bp) specific for the exon 1b estrogen receptor-α promoter sequence (GenBank accession number X98236; forward: 1942-AGAAAGCACTGGACATTTCT-1961). The oligonucleotides were radiolabeled (1 μl T4 polynucleotide kinase (PNK), Promega) with 5 μl [γ-^{32}P]ATP (Amersham) (2 h, 37 °C) and then hybridized to the membranes that were then subjected to autoradiography. Relative optical density (ROD) readings were determined using a computer-assisted densitometry program (MCID Systems; Imaging Research). To calculate the final signal for each sample, the ROD value of the band within the antibody lane (A) was divided by the ROD value of the band within the input lane (I). To control for equal loading between samples, the final signal of the exon 1_7 GR promoter, amplified from the acetyl-histone H3-K9 immunoprecipitations, was divided by the final signal from the β-actin promoter-α amplified from the same precipitate.

Intracerebroventricular infusions. Animals were anesthetized and secured in a stereotaxic frame, and a stainless-steel guide cannula (22 gauge), 8 mm length (Plastic One Inc.) was aimed at the left lateral ventricle (1.5 mm posterior to bregma, 2.0 mm lateral to midline, 3.0 mm ventral to the brain surface). After a 7-d recovery period, animals received a single infusion every day for seven consecutive days as described below. Animals were removed from their cages and gently held while an infusion cannula (28-gauge) attached to tubing (PE 20) was lowered into the guide. A total volume of 2 μl of TSA (100 ng/ml in DMSO) or DMSO vehicle alone was injected (using a Hamilton 10-ml micro-syringe) through the infusion cannula over a 1-min period. Infusion cannulae were left in place for an additional minute after infusion. Animals were then returned to their home cage.

Western blotting. Studies were performed as previously described[15]. Protein (40 μg, whole cell extract) rendered on nitrocellulose (C+, Amersham Pharmacia Biotech) was incubated with anti-rat GR-α monoclonal primary antibodies in blocking buffer (1:4,000; Affinity BioReagents) and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G antibody (1:3,000; Amersham Pharmacia Biotech). A single band was observed at ~92 kDa upon exposure to film (Amersham Pharmacia Biotech). To verify the accuracy of sample loading, selected blots were reprobed with a monoclonal antibody to tubulin (α-tubulin; 1:5,000; Biodesign International). A single band was observed at ~60 kDa and the intensity of the signal was similar in all lanes. Relative optical density (ROD) readings for the GR-α and α-tubulin bands were determined using a computer-assisted densitometry program (MCID 4.0; Imaging Research) from samples (n = 6 animals/group) run in triplicate on three different blots. The glucocorticoid receptor ROD value was divided by the α-tubulin ROD value to determine the final glucocorticoid receptor signal for each sample. For all studies, single blots were derived from samples from one animal.

HPA response to restraint stress. Animals were placed in Plexiglass restrainers (8.5 × 21.5 cm; Kent Scientific) for a 20-min period. Prestress blood samples were taken from rats within 30 s of removal from the home cage, and restraint stress was performed during the light cycle between 12:00 and 15:00 with blood sampling (300 μl) from the tail vein at 10, 20, 40, 60 and 90 min after the onset of restraint[12]. Plasma (10 μl) corticosterone was measured by radioimmunoassay (RIA) with a highly specific B antiserum (B3-163; Endocrine Sciences) and [^{3}H]corticosterone (101 Ci/mmol; NEN) tracer. The antiserum cross-reacts slightly with deoxycorticosterone (~4%) but not with aldosterone, cortisol and progesterone (<1%). The intra-assay and interassay coefficients of variation were 8.8% and 10.4%, respectively. The standard curve 50% effective concentration was 16 μg/dl, and the detection limit of the assay was 0.63 μg/dl.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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