Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus

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Early life experience is associated with long-term effects on behavior and epigenetic programming of the *NR3C1* (*GLUCOCORTICOID RECEPTOR*) gene in the hippocampus of both rats and humans. However, it is unlikely that such effects completely capture the evolutionarily conserved epigenetic mechanisms of early adaptation to environment. Here we present DNA methylation profiles spanning 6.5 million base pairs centered at the *NR3C1* gene in the hippocampus of humans who experienced abuse as children and nonabused controls. We compare these profiles to corresponding DNA methylation profiles in rats that received differential levels of maternal care. The profiles of both species reveal hundreds of DNA methylation differences associated with early life experience distributed across the entire region in nonrandom patterns. For instance, methylation differences tend to cluster by genomic location, forming clusters covering as many as 1 million bases. Even more surprisingly, these differences seem to specifically target regulatory regions such as gene promoters, particularly those of the protocadherin α, β, and γ gene families. Beyond these high-level similarities, more detailed analyses reveal methylation differences likely stemming from the significant biological and environmental differences between species. These results provide support for an analogous cross-species epigenetic regulatory response at the level of the genomic region to early life experience.

conservation

Variation in early life experience is associated with differences in life-long health and behavioral trajectories in animals as well as humans. For example, differences in maternal care in rats during the first week of life are associated with long-term effects on behavior and brain function that persist into adulthood, including alterations in the stress response (1). In humans, similar effects are observed. For instance, childhood maltreatment associates with development of both externalizing and internalizing personality traits and psychopathology in adulthood (2). The association in both rats and humans of stable developmental phenotypes with early life experience suggests that molecular mechanisms may serve as a memory of these early life experiences in both species. In fact, there is evidence that these long-term effects are, at least in part, mediated by epigenetic alterations in the brain. In particular, recent studies have found aberrant DNA methylation in the *NR3C1* (*GLUCOCORTICOID RECEPTOR*) gene promoter of the hippocampi of both rats and humans associated with differential early life experience (3, 4). Exposure of infant rats to stressed caretakers displaying abusive behavior produced persisting changes in methylation of the *BDNF* gene promoter in the adult prefrontal cortex (5). Early life stress in mice caused sustained DNA hypomethylation of an important regulatory region of the *AVP* gene (6).

Although explanations involving a single site are appealing, it is unlikely that the broad systemic response to early life experience would be associated with a few site-specific epigenetic changes. Indeed, we have previously shown that several hundred genes are differentially expressed in the hippocampi of adult rat offspring that received low compared with high maternal licking and grooming (LG) (7). Moreover, in the hippocampi of humans with documented childhood abuse, we have recently discovered methylation differences in the *rRNA* gene promoters that are scattered across the genome (8). Furthermore, recent evidence suggests that epigenetic regulation is not restricted to the few thousand bases around the transcription start sites of genes. Epigenetic changes associated with transcriptional changes can appear within the body of a gene (9) or even at high frequency across megabase-sized domains simultaneously deactivating dozens of neighboring genes (10, 11). These results led us to hypothesize that the epigenetic response to early life experience is not limited to a single gene promoter but that *NR3C1*, along with neighboring genes, might belong to a domain under coordinated control. To test this hypothesis, we recently investigated DNA methylation, H3K9 acetylation, and transcriptional profiles in a region encompassing 6.5 million base pairs centered at *NR3C1* in the hippocampus of adult rat offspring of high and low LG (12). We confirmed our hypothesis by identifying hundreds of robust DNA methylation differences between the offspring of high and low LG that were scattered across this large region.

Considering the parallel behavioral and epigenetic responses in humans and rats to early life environments described above, it is reasonable to assume that at least part of the broad epigenetic responses observed in rats to early life experiences may be evolutionarily conserved in humans. Therefore, in this study we investigated the extent of this conservation in humans by generating epigenetic profiles of the analogous region in humans, the 6.5 million base pair region centered at *NR3C1* (heretofore referred as the *NR3C1* locus). Such a cross-species investigation is further supported by the fact that there is an ∼35% sequence homology between the rat and human *NR3C1* loci, and that 80% of the genes in the human region have orthologs in the rat region.

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Data deposition: The DNA microarray data reported in this paper have been deposited into the Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo (accession no. ■■■).

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Results

Methylation Profiles in the *NR3C1* Locus of Rat and Human Hippocampi. We generated DNA methylation profiles in hippocampal samples obtained from the Quebec Suicide Brain Bank of 12 suicide completers with a history of severe childhood abuse and 12 nonabused controls. Profiles covered the genomic region from 3.25 Mb upstream to 3.25 Mb downstream of the *NR3C1* gene at 100-bp spacing and were created by using the method of methylated DNA immunoprecipitation (meDIP) followed by hybridization to a custom-designed Agilent 44K tiling microarray. Fig. 1 depicts the locus tiled with probes including the locations of genes along with estimated methylation levels and differences between the abuse group and controls. Previously published rat methylation profiles were generated using identical methods from the hippocampi of adult rat offspring of high and low LG and covering the synteneic region from 3.25 Mb upstream to 3.25 Mb downstream of the *NR3C1* gene at 100-bp spacing (12).

Conservation of the *NR3C1* Locus Gene Architecture. Overall organization of the *NR3C1* locus is significantly conserved, as shown by the almost identical order of orthologous genes across the locus (Fig. 2). Fig. 2, *Top* shows the positions of genes in human, and Fig. 2, *Bottom* shows their positions in rat. Fig. 2, *Middle* shows the rat–human "hybrid" created by assigning orthologous genes to positions similar to their relative locations in the human and rat genomes. Gray vertical lines in each panel coincide with transcription start sites. Black lines between adjacent panels link transcription start sites of orthologous genes in neighboring panels.

Expected DNA Methylation Patterns Confirmed. Methylation levels were estimated from microarray meDIP profiles by deconvoluting individual CpG methylation levels from the intensities of nearby probes (13). Estimates of these levels across the locus in human and rat are shown in Fig. 2 and are compared directly in the middle panel showing the human–rat hybrid. Overall, methylation levels seem to rise and fall in unison. Indeed, they do have a small but statistically significant correlation ($P < 0.0013$; $R = 0.048$). Given the regulatory role of DNA methylation, these patterns are unlikely to be random. For example, dips should correspond to active transcription start sites, CpG islands, and the 3′ ends of genes (14, 15). As shown for the rat profiles, we observed lower methylation levels around transcription start sites ($P \leq 1.68 \times 10^{-272}$, Wilcoxon rank sum test), at the 3′ ends of genes ($P \leq 9.9 \times 10^{-28}$), and inside CpG islands ($P \leq 10^{-300}$). In contrast to rat methylation levels, human methylation levels were lower near methylation-sensitive transcription factor binding sinks ($P \leq 0.06$). As the name suggests, methylation-sensitive transcription factor binding sinks are regions enriched for methylation-sensitive transcription factor binding sites as predicted by binding motifs. The lack of methylation decrease in these regions in rats is likely due to the fact that most transcription factor binding motifs have been derived from human studies rather than rat studies.

Conservation of a Widespread Methylation Response. Both the rat and human profiles revealed hundreds of differentially methylated regions (DMRs) associated with early life experience scattered unevenly across the *NR3C1* locus (Fig. 1). In total, there were 281 human DMRs, of which 126 had increased methylation in controls (cDMRs), and 155 had increased methylation in the individuals with histories of childhood abuse (aDMRs). Real-time PCR of meDIP samples was used to validate selected DMRs. We investigated 11 of these differences inside gene promoters located across the locus (Fig. 3). The rat profiles revealed more than twice as many DMRs (723), of which 373 were more methylated in high-LG offspring (hDMRs) and 350 were more methylated in low-LG offspring (lDMRs). This larger number in rat is possibly due to the greater genetic similarity and less environmental variability leading to increased power to detect differences within rat groups compared with human groups.

As observed in the rat profiles, the placement of the DMRs across the locus is nonuniform, resulting in large regions enriched with DMRs and others almost completely depleted of any DMRs (Fig. 1). In the sections below we explore these patterns in more detail.

Conservation of Long-Range Methylation Dependencies. In both species, DMRs showing the same direction of change according to environmental experience seem to form clusters covering large genomic regions, supporting a high-level organization linking distant sites. In general, there seem to be consistent dependencies between methylation differences as far apart from *NR3C1* as 1 million base pairs in both species (Fig. 4; figure 3a in ref. 12). As

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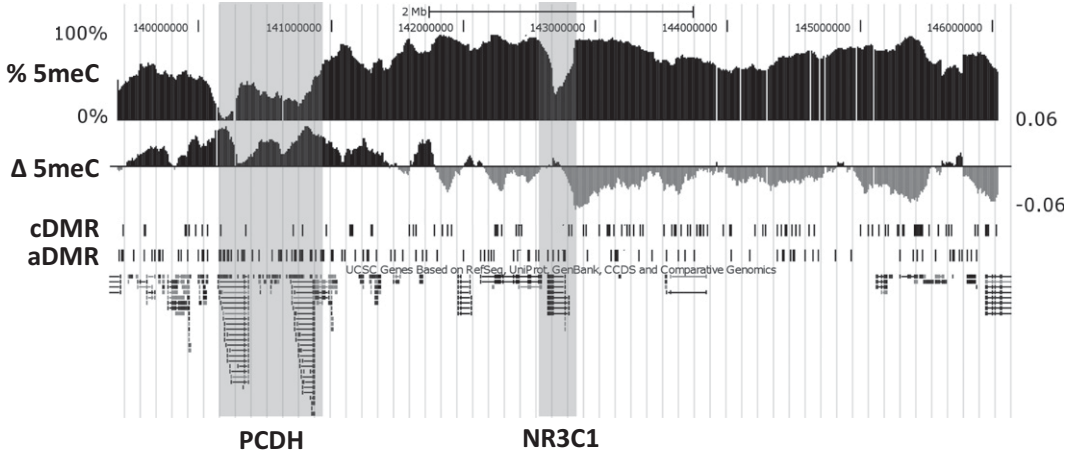

Fig. 1. Associations of human DNA methylation with early life abuse in the 6.5-Mb *NR3C1* locus. Track images obtained from the University of California, Santa Cruz genome browser (human genome assembly hg18) show % 5meC: average methylation levels across all samples estimated from microarray probe intensities; Δ 5meC: mean $\log_2$ fold differences between abused and control sample probe intensities, where positive values are shown in black and indicate higher methylation in abused samples, and gray values indicate higher methylation in control samples; cDMR: locations of cDMRs (significantly higher methylation in control samples); and aDMR: locations of aDMRs (significantly higher methylation in abuse samples). The locations of the protocadherin families of genes and *NR3C1* are identified by shading.

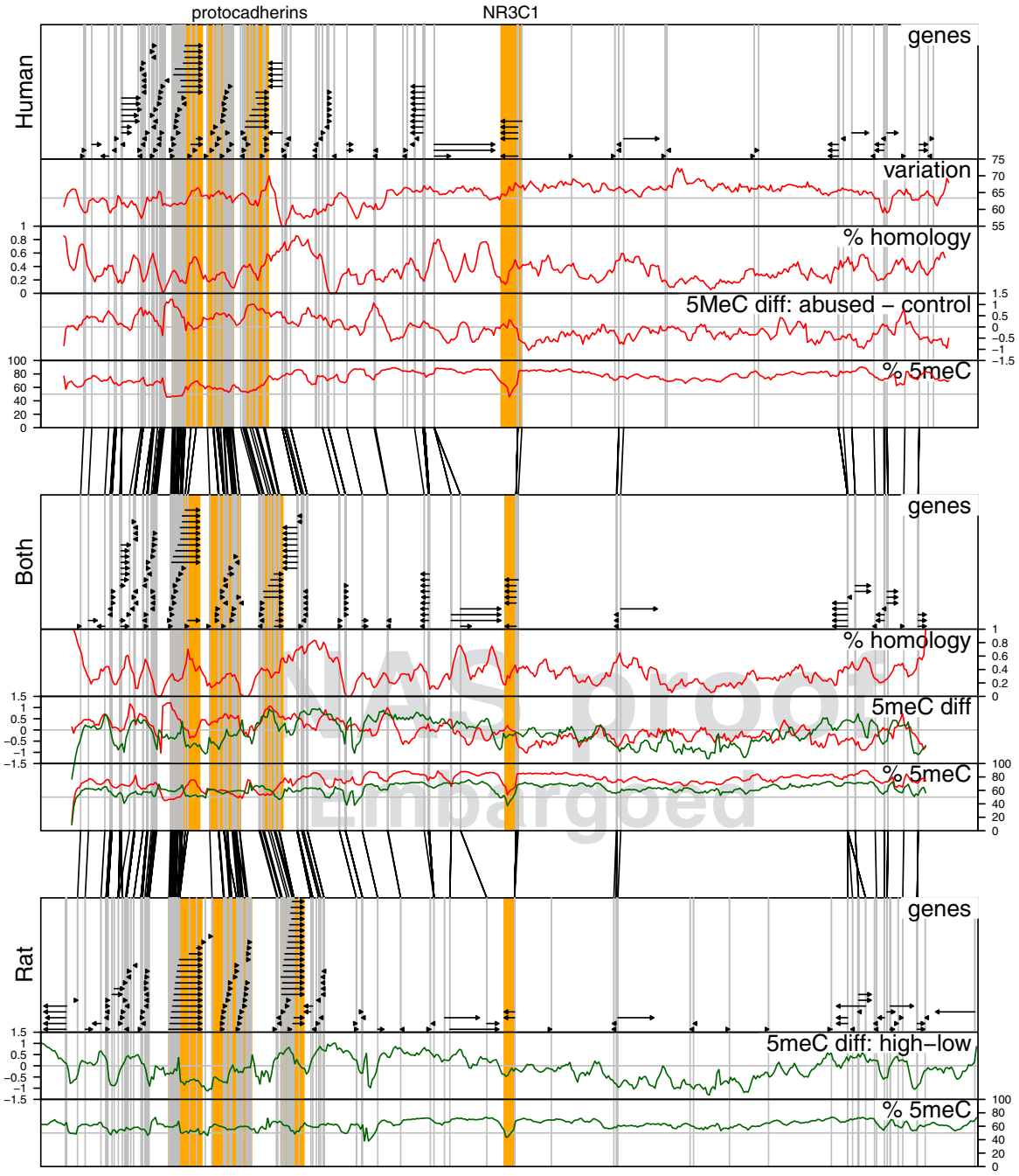

Fig. 2. Associations of human and rat DNA methylation with early life abuse in the 6.5-Mb *NR3C1* locus. *Top*, *Middle*, and *Bottom*: Each panel shows the 6.5-Mb NR3C1 locus. *Top*: Human locus. *Bottom*: Rat locus. *Middle*: Human–rat "hybrid" panel created by assigning orthologous genes to positions similar to their relative locations in the human and rat genomes. Each panel is divided into five labeled parts: genes: black horizontal arrows denote genes and the direction of mRNA synthesis; variation: graph indicates regions of high and low methylation variation across all human subjects; % homology: graph shows the percentage of bases in the human genome that were mapped by the lastz alignment tool to the rat genome; 5meC diff: graph shows mean $\log_2$ fold differences between sample groups (i.e., between abused and control humans and between high- and low-LG rats); and % 5meC: graph shows methylation levels estimated from microarray probe intensities. Across each panel, gray vertical lines demark transcription start sites. Black lines between panels link the positions of transcription start sites of orthologous genes. The lack of crossings between these lines illustrates conservation of gene architecture around NR3C1 between rats and humans.

an example of this long-range clustering, observe in Fig. 1 that if the human *NR3C1* locus is partitioned into two parts, one part left and the other part right of *NR3C1*, aDMRs are enriched in the left part ($P \leq 2.2 \times 10^{-32}$; hypergeometric), and cDMRs are enriched in the right part ($P \leq 2.2 \times 10^{-32}$). Partitioning the rat *NR3C1* locus in the same way, hDMRs are enriched in the left part ($P \leq 0.031$), and lDMRs are enriched in the right part ($P \leq 0.013$). To determine whether any of the rat DMRs were conserved in human, hDMR and lDMR sequences were mapped to the human genome using BLAT. In total, 111 of these sequences mapped successfully to the human locus; however, none of them overlapped with human DMRs. Interestingly though, just as hDMRs are enriched to the left and lDMRs are enriched to the right of *NR3C1* in rat, the mapped hDMRs are also enriched to the

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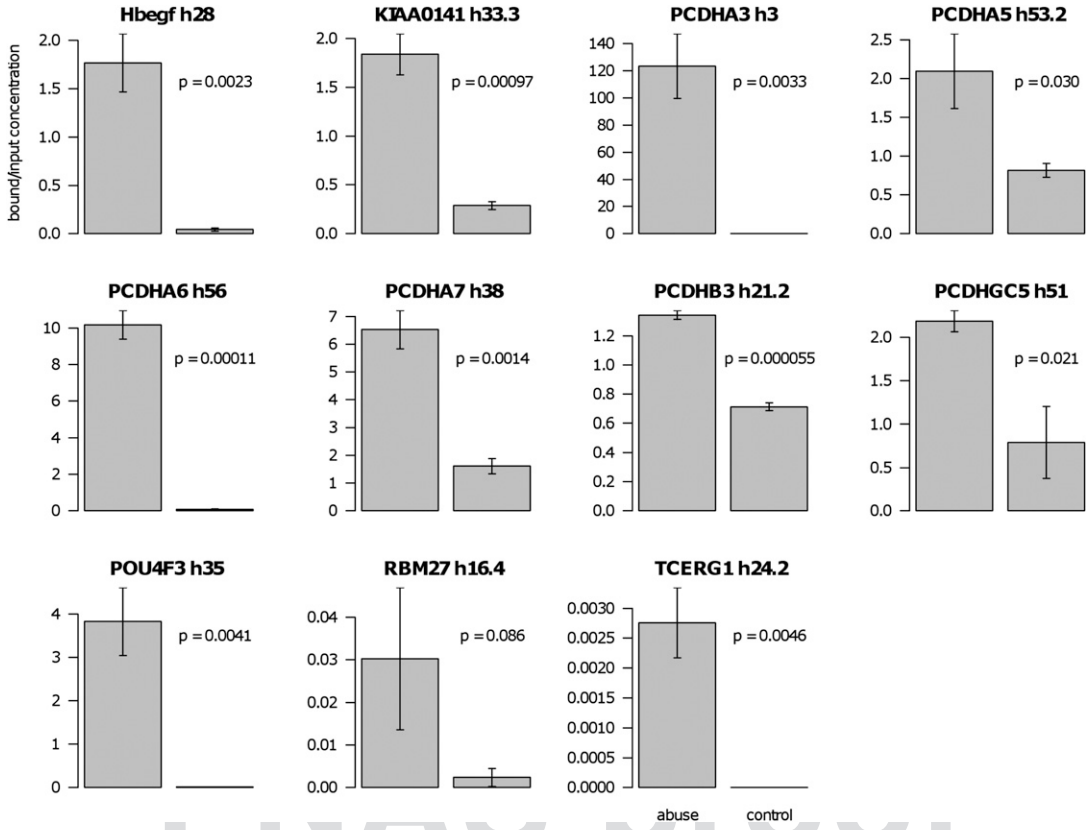

Fig. 3. Validation of microarray calls. Real-time PCR validation of microarray meDIP data is shown. Eleven of the 28 promoters identified as being differentially methylated by microarray (Table S1) were subjected to real-time PCR quantification of enrichment. The *y* axis represents concentration values generated by methylation-enriched and input DNA. *Left:* Concentration levels in the abuse group. *Right:* Concentration levels in the control group. Each real-time PCR was performed in triplicate. Error bars indicate SEM.

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left and the mapped lDMRs are enriched to the right of *NR3C1* in the human locus (Fig. S1). This suggests that change according to environment is conserved across species at a high level, although details about those changes differ between species.

Conservation of Enriched Methylation Response in Suspected Regulatory Sites. Given the regulatory role that DNA methylation plays, one might expect to see DMRs near known or suspected regulatory sites such as near transcription start sites, particularly coinciding with CpG islands, and transcription factor binding sites. Indeed, these regions tend to be enriched with DMRs in both rats and humans, although some of the details differ. Approximately 8% of DMRs in both rat and human intersect promoter regions (−2,000. . .+200 bp of the transcription start site; Table S1); however, whereas this intersection is statistically significant in humans ($P < 0.001$), it does not reach significance in rats ($P > 0.14$). When analogous regions at the 3′ ends of genes are included, the overlap of both human and rat DMRs is significant ($P < 0.001$ and $P < 0.0032$, respectively). In humans, this enrichment extends to 1,000 bp past transcription start sites ($P < 0.001$).

Interestingly, much of this enrichment in humans is explained by aDMR enrichment ($P < 2 \times 10^{-4}$, promoters; $P < 0.034$, 3′ ends of genes; $P < 2 \times 10^{-4}$, 1,000 bp after transcription start sites; $P < 0.019$, first exons) because cDMRs are depleted in nearly all of these regions ($P < 0.039$, promoters; $P < 0.031$, 1,000 bp after transcription start sites; $P < 0.02$, first exons). Supporting the regulatory nature of the sites targeted by aDMRs is the observation that they are enriched for methylation-sensitive transcription factor binding sinks ($P < 0.08$; *Methods*) and highly enriched with CpG sites ($P < 4.4 \times 10^{-14}$). Not surprisingly, cDMRS are depleted in these regions ($P < 0.02$) and depleted of CpG sites compared with the rest of the locus ($P < 1.4 \times 10^{-8}$).

In rat, such a simple characterization of lDMRs and hDMRs is not possible. lDMRs are enriched in some of these regions ($P < 0.02$, promoters; $P < 0.003$, 3′ ends of genes) but depleted in others ($P < 0.019$, 1,000 bp after transcription start sites; $P < 0.0011$, first exons). In contrast, hDMRs are enriched primarily in first exons ($P < 0.0008$) and, interestingly, also in last exons ($P < 0.0038$). On the other hand, depletion of lDMRs (rat) and cDMRs (human) are observed in last exons ($P < 0.0063$ and $P < 0.021$, respectively).

Differential Methylation Across *NR3C1*. We have previously shown that *NR3C1* gene expression is lower in the abuse group and that this decrease in expression associates with increased methylation levels in the promoter of a splice variant (1_F) of the *NR3C1* gene (4). The comprehensive mapping of the *NR3C1* locus presented here identified a total of seven DMRs in and around *NR3C1*: two upstream cDMRs, four aDMRs within the first and second introns, and one aDMR downstream of the gene (Fig. S2). The increased number of aDMRs compared with cDMRs is consistent with the repression of *NR3C1* in the abuse group. In rats there are similar DMRs throughout the gene, with the majority being lDMRs (figure 4a in ref. 12), also consistent with the repression of *NR3C1* in the low-LG group.

Conserved Methylation Sensitivity in the Protocadherin Families of Genes. Notable methylation differences in the *NR3C1* locus of both rats and humans are located downstream of *NR3C1* within the α-, β-, and γ-protocadherin (*PCDH*) gene clusters. All three clusters together are highly enriched for aDMRs ($P < 2 \times 10^{-4}$)

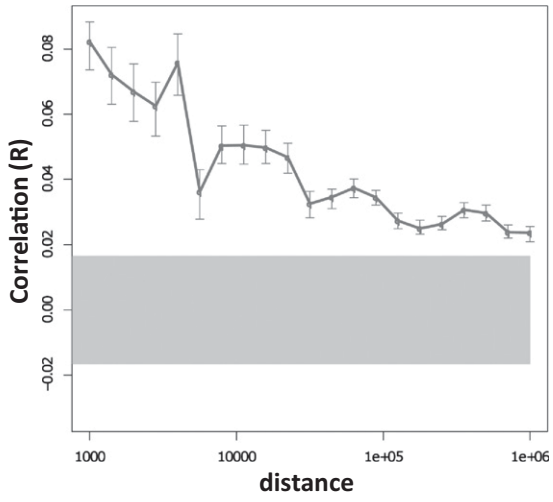

Fig. 4. Correlation of human DNA methylation associations with early life abuse in the 6.5-Mb *NR3C1* locus. Pearson correlations of DNA methylation differences between the subject groups at various genomic distances. Error bars show 95% confidence intervals for the correlation values. The gray highlight shows the expected 95% confidence interval if there is no correlation between methylation differences at different genomic sites. This confidence interval does not overlap with the error bars associated with distances less than 1 Mb, suggesting the existence of systematic dependencies between methylation differences at distances up to 1 Mb.

and depleted of cDMRs ($P < 0.0014$). Of the three clusters, α-*PCDH* is most enriched for DMRs ($P < 0.054$) and, particularly, for aDMRs ($P < 2 \times 10^{-4}$). Fig. S3 depicts the methylation differences within the *PCDH* gene clusters. Similarly to aDMRs, lDMRs are highly enriched in the *PCDH* gene clusters ($P < 0.01$).

These methylation differences observed in human hippocampus are of interest because the protocadherin families of genes are known to be regulated by promoter methylation (16–18) and have been implicated in synaptic function and neuronal connectivity (19–22).

Regions That Lack Differential Methylation. The existence of DMR clusters implies the existence of regions lacking methylation differences. For example, despite the fact that gene promoters are enriched with DMRs compared with other genomic regions ($P < 0.001$), only 28 of the 171 gene promoters (−2000…+200 bp around the transcription start site of a gene) contain a DMR. That leaves a lot of gene promoters unaffected by differential methylation, despite the fact that DMRs are widely distributed across the locus. For some reason, these promoters were "avoided." In fact, there are eight regions of more than 100 Kb within the *NR3C1* locus that contain no differentially methylated sites (Table S2). Permutation tests show that the expected number of such regions is only 2.2, with a maximum of six found in 1,000 such tests (DMR positions were randomly permuted within the locus). Three of the eight regions contained no genes, and one of the eight regions contained at least 10 genes, more than three times the number genes expected. Hence, these methylation profiles identify large gene-rich and gene-poor regions without any DMRs, evidence for a widespread but selective effect on DNA methylation levels within the *NR3C1* locus.

Discussion

There is growing evidence for association between variation in early life experience and differential methylation in several genes. Past studies focused on documented or highly predicted regulatory regions around the transcription start sites of these genes. Such an approach might miss important DMRs and ignore the larger scope of the DNA methylation response to environmental cues. Our recent study of the epigenetic response to maternal care in rats (12) determined that the epigenetic response is in fact not limited to a few sites but affects broader genomic regions. We asked here to what extent this broad response might be conserved across species. Hence, we performed a human study similar to our rat study covering the 6.5-Mb region centered at the *NR3C1* gene, wherein we examined differences in DNA methylation in the hippocampi of subjects who committed suicide and experienced severe abuse during childhood vs. control individuals with negative histories of abuse. Similarly to our rat study, we showed that differential methylation is not restricted solely to *NR3C1* promoters but instead appears at many sites throughout the *NR3C1* locus, both within as well distant from promoters (Fig. 1).

Although there are many methylation differences, they are not uniformly distributed across the locus, and our analysis describes several levels of structural organization of this association with early life experience showing surprising agreement with our parallel rat analysis. The differences in DNA methylation tend to concentrate in specific regions relative to transcription start sites and in specific regions containing dozens of genes within the *NR3C1* locus (Fig. 1), suggesting high-level organization. Especially remarkable was the discovery of a division of the entire 6.5-Mb locus into two major domains characterized by genomic sites with reduced DNA methylation in the abuse group upstream to the *NR3C1* locus and genomic sites with increased in DNA methylation downstream to the *NR3C1* locus (Fig. 1).

A strikingly significant number of DMRs can be found in the promoters of the protocadherin families of genes (Fig. 1) in both humans and rats, supporting the hypothesis that protocadherins play a key role in the response to early life experience. This hypothesis is consistent with previous findings that the complex expression patterns of the protocadherins are regulated by DNA methylation leading to differential promoter activation and alternative pre-mRNA splicing (16–18). That our methylation differences were observed in the hippocampus and that protocadherins have been implicated in synaptic function and neuronal connectivity (19–22) suggests that regional DNA methylation may play a role in concert with *NR3C1* changes in neuronal rewiring in response to early life experience.

The potential regulatory roles of the methylation differences that do not map to regulatory sites, such as transcription start sites and methylation-sensitive transcription factor binding sites, are more difficult to characterize. However, the conserved enrichment of methylation differences around these regulatory sites in humans and rats supports the existence of a regulatory role for other methylation differences yet to be elucidated.

Not surprisingly, given the important differences between rats and humans and the nature of their early life environments, comparison between the rat and human methylation changes associated with early life experience was neither simple nor straightforward. For example, the methylation profiles do not support a direct analogy at the individual base level between low maternal care in rats and childhood abuse in humans. However, such an analogy was not the purpose of our study. Instead, we reasoned that a cross-species comparison of DNA methylation associated with variation in early life environment would identify genomic regions beyond the promoters regions of *NR3C1* that are epigenetically labile in response to a range of early life experiences. Our results support this hypothesis. In both rats and humans, we identified a broad but selective response to early life experience that is enriched in suspected regulatory regions, exhibits evidence of a long-range coordination between distant sites, and seems to particularly target the regulation of the protocadherin families of genes, suggesting that these genes may also be involved in the response to early life experience. Such a conserved response motivates the development of novel

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experimental approaches to understand how these DNA methylation modulations affect genome function.

Methods

Methods related to the human samples only are provided here because methods and analyses related to the rat samples have already been published (12).

Ethics Statement. Studies with human subjects were approved by the McGill University institutional review board, and signed informed consent was obtained from next of kin. All procedures involving rodents were performed according to guidelines developed by the Canadian Council on Animal Care, and the protocol was approved by the McGill University Animal Care Committee.

Subjects and Tissue Preparation. Hippocampal samples obtained from the Quebec Suicide Brain Bank included 12 suicide subjects with histories of severe childhood abuse and 12 controls with validated negative histories of childhood abuse who did not differ in postmortem interval, sex, age at death, and brain pH (all $P > 0.05$). Psychiatric diagnoses were obtained by means of the *Structured Clinical Interview for DSM-III-R* (23) interview adapted for psychological autopsies, which is a validated method to reconstruct psychiatric and developmental history by means of extensive proxy-based interviews, as outlined elsewhere (24). To be considered in this study, all suicide subjects had to have a positive history of severe childhood sexual and/or physical abuse or severe neglect, as determined by most severe scores in the respective scales of the structured Childhood Experience of Care and Abuse (25) questionnaire adapted for psychological autopsies (26). Conversely, controls had to have validated evidence of negative lifetime histories of abuse and/or neglect.

All samples were from male suicide and control subjects of French-Canadian origin. Samples were dissected at 4 °C and stored in plastic vials at −80 °C until analysis. All samples were processed and analyzed blind to demographic and diagnostic variables. To be included in this study, all subjects had to die suddenly, with no medical or paramedic intervention and no prolonged agonal period. Suicide as the cause of death was determined by the Quebec Coroner's Office.

DNA Immunoprecipitation and Microarray Hybridization. The procedure for methylated DNA immunoprecipitation was adapted from previously published work (27–29). The amplification (Whole Genome Amplification kit; Sigma) and labeling reaction (CGH labeling kit; Invitrogen), and all of the steps of hybridization including washing and scanning were performed according to the Agilent protocol for chip-on-chip analysis. Microarrays were hybridized in triplicate for each sample.

Quantitative Real-Time PCR of Immunoprecipitated Samples. Gene-specific real-time PCR validation of microarray was performed for DNA methylation enrichment (30) for the same samples used for microarray experiments. Triplicate reactions were performed, and relative concentration was determined as a ratio of the crossing point threshold (Ct). The average concentration for each set of replicates was plotted along with its SEM. Primers for each amplicon are given in Table S3.

Microarray Design and Analysis. Custom 44K tiling arrays were designed using eArray (Agilent). Probes of ~55 bp were selected to tile all unique regions within ~3.25 MB upstream and downstream of the *NR3C1* gene described in Ensembl (version 44) at 100-bp spacing. Probe intensities were extracted from microarray scan images using Agilent's Feature Extraction 9.5.3 Image Analysis Software and analyzed using the R software environment for statistical computing (31). Background corrected log-ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray. Microarrays were normalized to one another using quantile normalization (32).

All genomic coordinates are given with respect to the hg18 human genome assembly.

In some cases, DNA methylation levels at genomic locations were estimated from microarray probe intensities. In these cases, a Bayesian convolution algorithm was used to incorporate probe values from nearby probes (13).

Differential methylation between groups was determined in two stages to ensure both statistical significance and biological relevance. In the first stage, linear models implemented in the "limma" package (33) of Bioconductor (34) were used to compute a modified *t* statistic at the individual probe level. An individual probe was called differentially methylated if the significance of its *t* statistic was at most 0.05 (uncorrected for multiple testing) and the associated difference of log-normalized means between the groups was at least 0.5. Given that the DNA samples were sonicated into 200- to 700-bp fragments before hybridization, we assumed that probes within 500 bp should have approximately similar probe scores. Therefore, in the second stage, we computed differential statistics for 1,000-bp intervals from the differential statistics of the probes that they contained. The intervals tiled the entire 6.5-Mb region under investigation at 500-bp spacing. Differential significance of these intervals was determined using the Wilcoxon rank-sum test comparing *t* statistics of the probes within the interval against those of all of the probes on the microarray. Significance levels were then adjusted to obtain false discovery rates. An interval was called differentially methylated if it satisfied each of the following: (*i*) its false discovery rate was at most 0.2, and (*ii*) the 1,000-bp interval contained at least one probe called differentially methylated. The first requirement ensured that several probes in the interval had similar group differences, and the second requirement ensured that the difference was not simply weakly distributed across the entire interval and consequently difficult to validate. Intervals satisfying these tests were called differentially methylated regions (DMRs). Consecutive DMRs for which the difference of means showed greater methylation in the abused group were called aDMRs and the converse cDMRs. Consecutive a/cDMRs were coalesced into single a/cDMRs.

Statistically significant enrichment or depletion of DMRs in specific regions such as CpG islands or gene promoters was computed using permutation tests on the locations of DMRs. More specifically, the statistic used the number of base pairs overlapping between DMRs and the regions in question, for example CpG islands. A distribution for this statistic was computed by repeatedly (1,000 times) randomly assigning theoretically possible coordinates (based on the locations of probes) to the DMRs and then calculating the overlap between the regions and the newly located DMRs.

Methylation-sensitive transcription factor sinks were computed by position weight matrices for specific transcription factors from the Transfac (35) and Jaspar (36) databases, including *AP2*, *CBF*, *CREB*, *ETS*, *FOXP3*, *GABP*, *GATA1*, *NF-kappaB*, *NGFIA/EGR1*, *NR3C1*, *P53*, *RUNX*, *SP1*, *SP3*, *TCF*, and *USF1*. There is evidence that the activity of each of these transcription factors is affected by the presence or absence of DNA methylation (3, 37–48). For some of these transcription factors, the databases contained multiple identical position weight matrices. To avoid having these matrices bias the identification of transcription factor sinks in favor of a single transcription factor, we removed one position weight matrix for any pair whose targets overlapped 75% of the time. Transcription factor targets were identified by scanning the sequence with a second-order position weight matrix adjusting log-likelihoods with the 500-bp sequence background context (49). Sites with a log-likelihood greater than 14 were called binding sites. To make the remaining computation to identify transcription factor sinks more efficient, the genome was then partitioned into 100-bp segments, and the binding score for each transcription factor in each segment was set to the maximum log-likelihood in that segment. Each segment was called a transcription factor sink if its transcription factor scores were significantly higher than average as determine by the Wilcoxon rank sum test ($P \leq 1 \times 10^{-9}$ or 6×10^{-5} after Bonferroni correction).

The variability of a probe was quantified as the number of sample pairs for which all normalized replicate log-ratios for one sample were at least 0.5 greater than all normalized replicate log-ratios for the other sample.

Overall homology between human and rat were computed using the lastz program (50). Specifically, the lastz program was used with default settings to align the human and rat *NR3C1* locus sequences. The percentage of homology was given as the percentage of human sequence that was successfully to the rat sequence.

Rat DMR sequences, all 1,000 bp long, were mapped onto the human *NR3C1* using BLAT (51) with the following settings: tile_size = 10, step_size = 10, min_match = 2, min_score = 400, min_identity = 75, and max_intron = 250.

Fig. 4 illustrates the correlation of methylation differences across various genomic distances as Pearson correlations of modified *t* statistics computed by limma for all pairs of probes at specified distances (with a 10% tolerance). Error bars denote 95% confidence intervals obtained from 1,000 bootstraps composed of randomly selected probe pairs with replacement. The gray rectangle denotes the 95% confidence interval for correlations of probe pairs independent of their distance. Independence was simulated by with 500 random permutations of the probe coordinates.

All microarray data are MIAME compliant, and the raw data have been deposited in the Gene Expression Omnibus.

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Maternal adversity and ecological stressors in natural populations: the role of stress axis programming in individuals, with implications for populations and communities

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Running Head: Ecological basis for maternal stress programming

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Summary

1. Biomedical researchers have long appreciated that maternal stressors can induce preparative and adaptive programming in offspring via exposure to maternal Glucocorticoids (GCs). However, few ecologists are aware of the capacity for maternal GC exposure to translate ecological and environmental stressors into preparative and adaptive programmed offspring responses in free-living systems. We review a growing body of experimental work indicating that circulating maternal GCs link ecological stressors with adaptive programming of the stress axis.
2. To encourage rigorous testing of this paradigm in a broad range of ecological systems, we introduce principal extrinsic stressors with a recognized potential to alter maternal circulating GC levels. We then explore what is known from the biomedical literature regarding the underlying physiological and epigenetic mechanisms of stress-induced programming of individual phenotypes to predict how variation in ecological stressors can produce individual variation in stress axis management.
3. To appreciate the potential evolutionary inertia (i.e., adaptive value) of this programmed individual variation, we review key recent studies in free-living systems that test its adaptive function, and then discuss how variation in stress-axis programming may scale up to influence populations and ecological communities.
4. Throughout, we emphasize that natural and human-induced ecological stressors play a fundamental role in programming the capacity of individuals, populations and communities to respond to both predictable and unpredictable ecological change via translating maternal adversity into responsive programming of the vertebrate stress axis. Given the huge potential, it is encouraging that ecologists are beginning to examine how and why maternal GCs translate ecological and environmental stressors into preparative stress axis programming in free-living systems.

Introduction

Ecologists are well aware that glucocorticoid ('stress' - GC) hormones have the potential to mediate the link between environmental variability and variation in the behavior, life-history strategies, and fitness of organisms (Wingfield and Sapolsky 2003; Boonstra 2005; Reeder and Kramer 2005; Wikelski and Cooke 2006; Romero et al. 2009; Love and Williams 2008a; Sheriff et al. 2009). Indeed, the mechanistic functioning of the hypthothalamus-pituitary-adrenal (stress) axis is highly conserved across vertebrate taxa, which underscores the biological importance of optimal GC management (Boonstra 2005; Wingfield 2005; Breuner et al. 2008). The release and management of circulating GCs plays two very important, evidently adaptive, biological roles in vertebrates: baseline GC levels maintain homeostatic energetic balance and are involved in normal day-to-day activities associated with the diurnal cycle (reviewed in Landys et al. 2006), while the acute, 'stress-induced' release of GCs mediates physiological and behavioral responses to environmental challenges (Breuner et al. 2008). Output from the stress axis begins with sensory input on environmental variation into the hypothalamus and ends with the release of GCs in the form of cortisol or corticosterone (Breuner et al. 2008). Given the important maintenance and response roles, variation in GC secretion is expected to be a major factor regulating the energetic and life history trade-offs that produce optimal investment decisions and ultimately drive variation in fitness (Hadany et al. 2006; Bonier et al. 2009). Determining how the interaction between intrinsic state and extrinsic environmental factors produces widespread, and apparently adaptive, intra-specific variation in the functioning of the stress axis is therefore an important goal for evolutionary and physiological ecologists (Love et al. 2009; Sheriff et al. 2010).

Related questions have been a focus of interest by biomedical researchers studying mechanisms conferring inter-individual variation in disease susceptibility (McGowan and Szyf 2010b). In the human literature, epidemiological studies have provided considerable evidence of the capacity of environmental factors during early life to alter health trajectories (Low, Gluckman and Hanson 2012). Barker's 'thrifty

phenotype' hypothesis proposed that maladaptive outcomes were the result of a mismatch between conditions of low food availability during development and actual environmental conditions of adequate nutrition (Hales and Barker 1992). This proposal stimulated considerable research into responses in humans to a range of environmental conditions during development that influence human health trajectories in a manner consistent with that of an adaptive response, chief among them effects of nutrition and parental care (Low, Gluckman and Hanson 2012; Gluckman et al. 2005a). Biomedical studies of humans and laboratory animals indicate a profound effect of parental care early in life on the epigenetic programming of the stress axis and associated behaviours (McGowan et al. 2008, 2009,2011).

The capacity for maternal GC exposure to translate ecological and environmental stressors into preparative and adaptive programmed responses in offspring (size, growth, and performance) has been well documented in free-living systems across four diverse taxa (birds: Love et al. 2005, Love and Williams 2008b; mammals: Sheriff et al. 2009, 2010; reptiles: de Fraipont et al. 2000, Meylan et al. 2002, Meylan and Clobert 2005; fish: McCormick 1998, 1999, 2006). Maternal stress can also significantly alter the ability of offspring to respond to future ecological stressors via programming effects on the stress axis (Hayward et al. 2006; Love et al. 2008; Sheriff et al. 2010; Haussmann et al. 2011), something that medical and laboratory mammalian researchers have long appreciated given that the embryo/fetus and post-natal offspring must balance immediate physiological and developmental challenges within the light of preparing for adult life (reviewed in: Seckl 2001, 2004; Seckl and Meaney 2004; Gluckman et al. 2005b; Macri and Wurbel 2006; Meaney et al. 2007). Contemporary experimental research suggests that a diverse array of ecological stressors during reproduction can phenotypically alter the stress-axis of offspring via exposure to maternal stress during pre- and post-natal development: environmental effects on maternal state (Love and Williams 2008a), predation pressure (Sheriff et al. 2010), quality of the rearing environment (Love et al. 2003; Pravosudov and Kitaysky 2006) and even the unpredictability of the social environment (Landys et al. 2011). Moreover, permanent programming of the stress axis (as opposed to reversible developmental

105 flexibility, i.e., Lendvai et al. 2009) suggests that effects are not just unavoidable
106 developmental costs, but rather adaptive responses that prepare individuals to
107 behaviorally cope, reproduce and survive in environments where ecological stressors
108 are frequently encountered or are greater in intensity (Meylan and Clobert 2005; Love
109 and Williams 2008b; Preisser 2009; Sheriff et al. 2010).
110 Here we review a growing body of experimental research testing the hypothesis
111 that circulating maternal GCs link ecological stressors with adaptive programming of the
112 vertebrate stress axis in free-living systems. To encourage rigorous testing of this
113 hypothesis in a broad range of ecological systems, we briefly review extrinsic stressors
114 with a recognized potential to alter maternal circulating GC levels. We then explore how
115 pre-natal exposure to maternal GCs, or to GC-altered post-natal maternal behavior,
116 affects the underlying physiological and epigenetic mechanisms driving stress-induced
117 programming of individual phenotypes. We then examine how variation in ecological
118 stressors can result in individual variation in the stress axis. To understand the
119 evolutionary role of this programmed variation, we review recent work testing its
120 adaptive function to predict how individual variation in stress-axis programming can
121 scale up to influence populations and ecological communities. Throughout, we hope to
122 emphasize that to appreciate the ecological causes of evolution (*sensu* MacColl 2011),
123 especially within light of increasingly rapid human-induced alterations to ecosystems,
124 ecologists must understand the underlying mechanisms generating individual variation
125 in the capacity to respond to both predictable and unpredictable ecological change.
126
127 **1. Ecological and environmental variation as maternal stressors**
128 Numerous ecological stressors are predicted to affect maternal GCs and thus influence
129 the programming of the offspring stress axis. Many of these extrinsic variables are those
130 ecologists routinely study (e.g., predation risk, resource availability, social interactions),
131 whereas some are novel emerging stressors (e.g., climatic variability and climate
132 change, human disturbance). In studies of the ecological stressors that influence
133 maternal GC levels, and therefore offspring, few researchers routinely measure GC
134 levels from pre-breeding, pregnant or gravid females in free-living systems (Love et al.

2009). Moreover, early studies linking GCs and reproduction focus almost exclusively on males (see Williams 2008). Traditionally therefore, less focus has been placed on maternal GCs during the stages when programming of the offspring stress axis is expected to occur. However, there are a number of strong emerging examples linking key ecological stressors to maternal GCs and offspring programming in a wide variety of free-living model systems.

Predation Risk and Resource Availability

Two of the most significant environmental factors affecting organismal populations are predation and access to resources (Krebs et al. 1995; Clinchy et al. 2004; Sheriff et al. 2011). Ecologists have long theorized about the link between predation risk and physiological stress, and both risk and direct exposure elevate GCs in free-living vertebrates (rev. in Hawlena and Schmitz 2010; Clinchy et al. 2012). Predation risk has been shown to increase maternal GC levels in particular in a variety of free-living taxa. In mammals, an increase in the number of predators, or the risk of predation, has been demonstrated to increase maternal GC levels at both an individual and a population level (snowshoe hares – Boonstra et al. 1998; Sheriff et al. 2010, 2011; yellow-bellied marmots - Monclús et al. 2011). In birds, an increase in nest predation, perceived risk of predation and direct exposure to predators has been shown to increase maternal GC levels, or GC secretion into eggs (barn swallows – Saino et al. 2005; European starlings – Love et al. 2008; song sparrows – Travers et al. 2010). In fish, an increase in the number of egg predators, or an experimental elevation in predation risk, increased both maternal GC levels and GC secretion into eggs (tropical damselfish - McCormick 1998; sticklebacks – Giesing et al. 2011).

Not surprisingly, the quantity, quality and predictability of resources can also act as ecological stressors in mothers (Love et al. 2005), given the significant role that GCs play in managing energetic balance at the level of the individual (Landys et al. 2006). Biologically relevant, unpredictable changes in food availability are known to increase maternal or female GC levels in both free-living birds and mammals (Kitaysky et al. 1999, 2007; Benowitz-Fredericks et al. 2008; Shultz and Kitaysky 2008; Jeanniard du

Dot et al. 2009; Welcker et al. 2009), as is a reduction in the energetic and micronutrient quality (rather than quantity) of resources (Chapman et al. 2007; Dantzer et al. 2011). A reduction in access to resources via competition can also reduce female quality and increase maternal GC levels (de Fraipont et al. 2000; Meylan et al. 2002). More often than not studies have linked the outcome of reduced resource quality/availability (i.e., low or declining body condition) to elevated maternal GC levels during egg laying or pregnancy (de Fraipont et al. 2000; Meylan et al. 2002; Love et al. 2005, 2009; Monclús et al. 2011). Although less well understood, resource availability and predation risk can act synergistically to increase maternal GC levels (Sheriff et al. 2010), with interactive effects often being much stronger than predicted from studying their effects in isolation (Clinchy et al. 2004). Finally, reduced resources and declining maternal body condition can also affect post-natal maternal investment in offspring (i.e., reduced provisioning) via an increase in maternal GCs (Love et al. 2004; Angelier et al. 2007, 2009).

Social Interaction

Social interactions, conflicts and dominance relationships have long been known to act as environmental modulators of circulating GC levels in vertebrates (Sapolsky et al. 2000; Creel 2001; Creel et al. 2012). In social mammals, subordinate reproductive females often exhibit high GC levels compared to dominant reproductive females (Sapolsky et al. 2000; Creel 2001). However, in cooperatively breeding mammals, dominant females generally have higher GC levels (Creel 2001; Koren et al. 2008; although see Young et al. 2006). Furthermore, aggressive interactions, or even the perceived presence of increased competition via the visual presence of a conspecific, have been shown to increase maternal GCs, and therefore GCs deposited into the eggs, in tropical reef fish species (McCormick 1998, 1999, 2006). In free-ranging female morphs of the side-blotched lizard, individual, reproductive females exhibit different GC levels in relation to the dominance status of their nearest neighbor (Comendant et al. 2003). Finally, semi-colonial breeding female European starlings nesting away from conspecifics deposited increased levels of GCs into eggs compared to females nesting in close association with conspecifics (Love et al. 2008). Clearly, social interactions

have the potential to influence maternal GC levels and offspring programming and the adaptive advantages of such programming will greatly rely on the social structure and interactions between conspecifics.

Habitat Quality, Human Disturbance and Climate Change

A degradation of habitat integrity and increases in human disturbance are predicted to increase circulating maternal GCs (Madliger et al. 2011). With respect to maternal stress, declining habitat quality has been shown to increase maternal GC levels in a variety of free-living taxa. Reductions in wintering habitat quality of migratory species can increase maternal GCs at arrival on breeding grounds (Marra and Holberton 1998) and large-scale geographic reductions, or variability in resource abundance/quality, can influence maternal GC levels during the pre-breeding stage (Kitaysky et al. 1999, 2007; Shultz and Kitaysky 2008). Human disturbance, both as recreational and industrial activity, can also cause an increase in maternal GCs in mammals (Creel et al. 2002; Wasser et al. 2011) and birds (Thiel et al. 2008; Zhang et al. 2011). Although relationships between habitat integrity and maternal GCs are often highly complex and may be mediated via effects on resource availability or other environmental factors (Madliger et al. 2011), a decline in habitat quality appears to be consistently related to elevated maternal GC levels.

Ecological physiologists have shown that variation in temperature, humidity and wind speed can all cause increases in stress-induced GCs in vertebrates, although the degree of this response can depend on resource availability and how well individuals are acclimated to conditions (Wingfield et al. 1998; Romero et al. 2000; Breuner and Hahn 2003). However, current data linking climatic variation and GCs during the early stages of reproduction is heavily male-biased (i.e., Wingfield et al. 1998; Breuner and Hahn 2003). Sheriff and colleagues (2012) found that differences in the timing of snowmelt and spring conditions may alter seasonal patterns of GC secretion in free-living arctic ground squirrels, with later snowmelt prolonging elevated GC levels in spring. Furthermore, unpredictably high precipitation and cooler temperatures were linked with elevated GC levels in these animals. Until recently, the effects of climate

change on maternal GC levels were only explored theoretically (i.e., Boonstra 2004; Wingfield 2008). Thankfully, an increasing diversity of emerging work is proposing to examine the physiological mechanisms linking individuals to their environment (i.e., Love et al. 2010; Sheriff et al. 2012; Wingfield 2012), and we expect studies linking climate change, maternal stress and offspring programming to increase in the coming years.

2. Mechanisms by which maternal stress can be transferred to offspring

Maternal stress results in life-long changes in stress axis function and behaviour in offspring across a large variety of taxa and maternal GCs are the primary candidate mediating such programming (mammals – Meaney et al. 2007, Sheriff et al. 2010; Monclús et al. 2011; birds - Hayward and Wingfield 2004, Saino et al. 2005, Love and Williams 2008b; fish - McCormick 1999, 2006; reptiles - de Fraipont et al. 2000, Meylan et al. 2002, Meylan and Clobert 2005). However, the method of birth (placental vs. egg-laying) and the timing of maturation of the HPA axis relative to birth are important considerations in understanding the mechanisms by which maternal stress may program the offspring's brain.

In egg-laying vertebrates, embryos are exposed only to those maternal hormones deposited in the egg during the relatively short period when the yolk is being produced. Both experimental and predator-induced increases in maternal GCs during laying can increase GC concentration in the yolks and albumin of eggs (Hayward and Wingfield 2004, Love et al. 2005, Saino et al. 2005). Presently, little is known about the mechanisms of GC transfer between the mother and the egg (Groothuis et al. 2005), although there appears to be a positive correlation between maternal and yolk GC levels in at least one species (Love et al. 2005). Changes in maternal care and provisioning in early life may also greatly affect stress axis function and behavior in egg laying vertebrates. For example, in black-legged kittiwakes a 20-day food restriction during development resulted in a subsequent increased GC levels in 30 day old chicks (Kitaysky et al. 1999). In European starlings, reducing maternal provisioning rates

increased the responsiveness of the axis in offspring, especially female fledglings (Love and Williams 2008b).

In mammals, the timing of maturation of the HPA axis relative to birth is highly species specific and in animals that give birth to precocial young (sheep, guinea pigs, hares) maximal brain growth and maturation takes place in utero (Dobbing & Sands 1979). In contrast, in animals that give birth to altricial young (rats, rabbits) much brain development occurs in the immediate postnatal period (Dobbing & Sands 1979). Thus, the timing of an increase in maternal stress will greatly impact animals differentially depending upon the species involved. Evidence for the specific mechanisms of fetal and neonate programming comes from the biomedical, mammalian literature and is termed prenatal and postnatal programming and we will discuss as such.

Prenatal Programming

In laboratory mammalian studies, there is a large and growing body of research indicating that maternal stress during the later stages of gestation results in life-long changes in stress axis function and behaviour in offspring (Matthews et al. 2004; de Kloet et al. 2005; Owen et al. 2005; Meaney et al. 2007). GCs are essential for normal brain development, exerting a wide range of organizational effects via the glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) in the brain (Matthews 1998). However, sustained exposure to, or removal of, GCs during development can permanently alter brain structure and function (Sapolsky 1987; Muneoka et al. 1997; Matthews 2002). Pre-natal exposure to GCs causes a decrease in GR and MR in the hippocampus, leading to a weaker negative feedback of the stress axis and elevated levels of GCs in adult offspring (Levitt et al. 1996; Welberg et al. 2001; Welberg & Seckl 2001; Emack et al. 2008; Fig. 1).

Under normal conditions exposure of the mammalian fetus to endogenous maternal GCs is restricted by placental expression of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2; Burton & Waddell 1999; Seckl 2004). 11 β -HSD2 interconverts GCs (cortisol and corticosterone) to the inert forms cortisone and 11-dehydrocorticosterone (DH-B; Funder 1996). However, when mothers are exposed to a

stressor, placental expression of 11 β -HSD2 decreases or fails to increase (Lesage et al. 2001; Lucassen et al. 2009) meaning that offspring may have little capacity to buffer their exposure (or they are not responding because it is adaptive not to buffer). Since maternal GC levels are much higher (10-fold in guinea pigs; Owen et al. 2005) than those of the fetus, subtle changes in 11 β -HSD2 activity may have profound effects on fetal GC exposure.

The mechanisms by which fetal exposure to GCs alter brain development remain poorly understood. However, accumulating evidence points to altered epigenetic mechanisms, by which experiences 'program' long-term changes in gene expression in the absence of changes in DNA sequence (McGowan & Szyf, 2010a, 2010b; Szyf, McGowan & Meaney, 2008). Laboratory experiments in rodents have shown that the physiological and behavioural alterations associated with prenatal stress are accompanied by transcriptional and epigenetic alterations in the brain in genes involved in HPA axis regulation, including altered DNA methylation in promoter regions of the GR and corticotrophin receptor genes (Mueller & Bale, 2008). DNA methylation is the best-studied epigenetic mark, its presence in gene promoters is usually associated with transcriptional silencing. Thus, prenatal programming effects derive from environmentally induced alterations of materno-fetal signaling, involving systems that determine fetal GC exposure. Ultimately, increased maternal adversity and GC levels result in an increase in fetal GC exposure and a permanent decrease in GR expression, which in turn leads to greater GCs levels in the offspring.

Postnatal Programming

Maternal influences during the very early postnatal period can also effect GR expression and offspring behaviour (Francis & Meaney 1999; Meaney 2001; Meaney et al. 2007). Evidence of postnatal programming dates back to studies by Levine and Denenberg during the 1950's who found that brief periods of neonate handling (resulting in greater maternal care) decreased offspring stress responses to stressors in mice and rats. More recently in rats, adult offspring of mothers who naturally exhibit high levels of care show elevated hippocampal GR expression, enhanced negative feedback sensitivity and a

more modest response to stressors (Liu et al. 1997; Fig. 1). As adults these offspring also display high maternal care themselves (Meaney 2001). Cross fostering the biological offspring of high and low caring mothers on the first day of postnatal life reverses this phenotype (i.e., the offspring phenotype matches that of the mother that raised it, not its biological mother) suggesting a direct relationship between maternal care and the development of the HPA axis and behaviour (Francis et al. 1999).

Weaver and colleagues showed that maternal care altered DNA methylation in the offspring at a GR gene promoter in the hippocampus by inhibiting the binding of NGFI-A, a transcription factor that drives GR expression (Weaver et al. 2004, 2005, 2007; Fig. 2). In this case, the presence of DNA methylation at sites recognized by NGFI-A inhibited the binding of the transcription factor, leading to reduced mRNA expression. These results imply that increased DNA methylation of GR promoter leads to fewer GRs, a less rapid response to stress, and a slower recovery after the stressor is over. Sequences within the GR promoter showed lower levels of methylation in offspring of high caring mothers, while those sites in offspring of low caring mothers showed relatively higher levels of methylation. These differences emerged within the first week of life, were reversed with cross-fostering, and persisted into adulthood. Infusion with the histone deacetylase inhibitor Trichostatin A (leading to a relatively open chromatin configuration and generally increasing transcription) into the brain of low care offspring or infusion of methionine (a methyl donor which increases DNA methylation in the presence of methyltransferase enzymes) into the brain of high care offspring eliminated group differences in DNA methylation pattern, the binding of NGFIA to the GR promoter, GR expression and HPA responses to stressors. More recently, McGowan et al. (2011) found evidence of wide spread but specific epigenetic and transcriptional alterations of the GR gene extending far beyond the GR promoter associated with differences in maternal care. A number of other groups have also found evidence of epigenetic regulation in the brain by altered parental care or stress-related early adversity (e.g. Murgatroyd et al., 2009; Roth et al., 2009). Thus, there is mounting evidence that epigenetic mechanisms coordinate wide spread changes in gene expression in response to differences in early maternal care or adversity.

344 Postnatal programming effects derive from environmentally induced alterations of
345 materno-neonatal interactions, involving systems that determine methylation patterns of
346 GR gene promoter sequences and additional loci. Increased maternal care (resulting
347 from mothers with lower GC levels) results in decreased methylation of the GR promoter
348 and increased GR expression, which in turn leads to lower GC levels in adult offspring.
349
350 **3. Programming of individual offspring phenotypes**
351 Individual variation in the output of the stress axis is one of this system's hallmarks
352 across a diversity of vertebrate taxa (see Williams 2008) and yet we know very little
353 about how this individual variation is mechanistically derived. Both inter-individual (i.e.,
354 differential exposure across mothers; Love et al. 2005; Love et al. 2009; Sheriff et al.
355 2010) and intra-individual (i.e., differential exposure across offspring for a given mother;
356 Love et al. 2008, Love and Williams 2008) variation is expected to produce significant
357 individual variation in the functioning of the stress axis of offspring. Sheriff and
358 colleagues (2010) found that GC levels of pregnant snowshoe hares were directly
359 echoed by that of their offspring, with entire litter groups reflecting the pattern of their
360 mothers at the time the young were born (Figure 3). Moreover, elevated maternal fecal
361 GC levels correlated with a heightened responsiveness in their progeny to further
362 stressors. Manipulative studies in birds also indicate that exposure to maternally-derived
363 GCs can contribute to variation in the stress reactivity of offspring (Hayward et al. 2006;
364 Love and Williams 2008). Love and Williams (unpubl. data) also found that within-nest
365 increases in the predicted exposure to maternal GCs (i.e., intra-clutch variation in
366 maternal GC exposure; Love et al. 2008) negatively correlated with the responsiveness
367 of the offspring stress axis (Figure 4). Moreover, a biologically relevant increase in
368 maternal GC exposure only further reduced the response of offspring exposed to the
369 lowest predicted maternal stress (Love and Williams unpubl. data; Figure 4). Laboratory
370 experiments in rats have shown that there is substantial within litter variation in maternal
371 care. Later in life (i.e., as mature adults) this difference is associated with differential
372 behavioural responses in experiments measuring stress-related behaviours (Van
373 Hasslett et al., 2011). Therefore, despite siblings having a similar genetic and rearing

environment, they may still face differential exposure to maternal GCs or maternal care and therefore exhibit variability in programming of the stress axis, potentially due to differences in background GC levels or even receptor density/location during development. Unfortunately in free-living mammalian populations, although there is also much variation in HPA responsiveness within a litter (Sheriff et al. 2010), no study to date has investigated causality of birth order or uterine placement. Although more studies are necessary, individual programming of stress axes via maternal GC exposure may play adaptive response roles to variation in future ecological stressors.

4. Adaptive role of programmed phenotypes

Optimal functioning of the HPA axis has long been considered paramount to maximizing fitness in vertebrates (Wingfield et al. 1998; Boonstra 2004; Wingfield 2005; Romero et al. 2009). Certainly, the vertebrate stress axis shows all the potential features of an adaptive trait: large intra-specific (individual) variation (Williams 2008); repeatability under consistent conditions (Ouyang et al. 2011); is considered heritable (Bartels et al. 2003; Federenko et al. 2004; Evans et al. 2006; Solberg et al. 2006); and has been selected upon in captivity (Satterlee and Johnson 1988; Evans et al. 2006). Indeed, a number of studies have shown that variation in the responsiveness of the stress axis plays adaptive roles when individuals are faced with changes in their ecological surroundings (i.e., Wingfield and Hunt 2002; Breuner and Hahn 2003). We also know that individuals with lower responses tend to be less affected by disturbance and show reduced rates of reproductive abandonment (Silverin 1998; Holberton and Wingfield 2003; Love et al. 2004; Angelier et al. 2009). Moreover, variation in HPA axis responsiveness in offspring (Cavigelli and McClintock 2003; Blas et al. 2007) and adults (Angelier at al. 2010) has been correlated with survival in vertebrates (Breuner et al. 2008). Finally, experimental manipulations of maternal stress are known to alter HPA axis functioning of exposed offspring in a number of non-biomedical systems (Hayward et al. 2006; Love and Williams 2008; Sheriff et al. 2010; Haussmann et al. 2011). How much information exists regarding how maternal programming of the stress axis influences the reproductive success and survival of offspring?

We generally lack data on how the programming of HPA activity *directly* affects offspring fitness in free-living species since few studies have performed manipulations of maternal GC exposure and then followed offspring into adulthood. However, data from studies of reproductive output in mothers, immediate (developmental) survival of offspring and proxies of fitness (growth, body size) allows for some predictions. Programming by maternal GCs is expected to influence offspring fitness through complex trade-offs between investment in development, reproduction and survival. Not surprisingly then, exposure to maternal GCs result in decreases in initial offspring body size and weight during early development and lower reproductive output for mothers of free-living species (Meylan and Clobert 2005; Love et al. 2005; Saino et al. 2005; Love and Williams 2008; Sheriff et al. 2009). However, a stress-induced reduction in initial maternal investment and overall output can benefit remaining offspring in the longer term through a reduction in developmental competition (Love et al. 2005; Love and Williams 2008; Breuner 2008), as well as beneficially influencing both dispersal (de Fraipont 2000; Meylan et al. 2002) and anti-predator behaviour in offspring (Meylan and Clobert 2005; Uller and Olsson 2006; Chin et al. 2009; Giesing et al. 2011).

Recently, maternal programming has been proposed to act as a bridge between the maternal and offspring environment (Love et al. 2005, Breuner 2008, Love and Williams 2008, Sheriff et al. 2009, 2010). However, to appreciate both the potential influence and direction of this relationship, it is critically important to examine phenotypic adjustments within the immediate environmental context in which they occur, as well as the longer-term environmental context that offspring face as reproductive adults (Love et al. 2005; Love and Williams 2008; Sheriff et al. 2009, 2010). In circumstances when maternal signaling is a reliable predictor of the offspring's future environment, maternal programming may be considered adaptive, increasing offspring fitness (i.e., Love et al. 2005; Love and Williams 2008; Chin et al. 2009). However, if maternal signaling is a poor predictor of the offspring environment, maternal programming may be maladaptive, negatively affecting offspring fitness (i.e., Sheriff et al. 2009, 2010). Since the consequences of maternal programming at the individual level are ecologically context

specific, they therefore have different potential ramifications for how individual responses scale up to influence populations and communities.

5. Scaling maternal programming up to populations and communities

Although maternal programming acts at the individual level it has the potential to greatly influence population dynamics by acting on factors such as reproduction, survival and dispersal. For example, maternal programming plays a large role in snowshoe hare cyclic population changes (Sheriff et al. 2009, 2010, 2011). During the decline phase, the high risk of predation increases maternal GCs and results in a decline in litter size, and offspring birth weight and size, while increasing baseline GC levels and offspring's stress responses. These effects persist into adulthood, likely lowering adult-offspring reproduction. The lower reproductive output would decrease the time necessary for foraging and thus may increase maternal survival (and thus maternal and offspring fitness). The increase in offspring GCs (and anti-predator behaviors associated with prenatally elevated GC levels: Drake et al. 2005; Emack et al. 2008) would increase offspring survival. Thus, during the decline phase although maternal programming may decrease reproduction it would result in higher maternal and offspring survival. Potentially allowing some individuals to escape the devastating predation effects and survive the collapse of the population. At the end of the decline phase and beginning of the low phase (when mothers experience high predation risk but offspring do not) the trade-off between a decrease in reproduction and an increase in anti-predator behaviors would be very costly. Thus, maternal programming may have a large influence on population dynamics depending upon the balance between the negative impact on reproduction and the positive effect on survival in an environmentally-context dependent manner.

Maternal programming via the stress axis can also affect offspring's propensity to disperse, but dispersal decisions may be the result of a complex interplay between maternal GC levels and maternal state (de Fraipont et al. 2000; Meylan et al. 2002; Meylan and Clobert 2005). For example in common lizards, Meylan et al. (2002) found that increased maternal GCs decreased dispersal in those offspring born to corpulent

463 mothers. High maternal GCs in less corpulent mothers resulted in increased offspring
464 dispersal. In other species, plasma GC levels in juveniles have also been found to affect
465 dispersal (Wingfield 1994; Silverin 1997). In willow tits, experimentally increased GC
466 levels enhanced dispersal rates; however, similar to lizards this was context dependent.
467 GCs only increased dispersal during a period of flock establishment (July – September);
468 however, when permanent winter flocks had become established, increased GC levels
469 had no effect on dispersal. Thus, maternal programming may influence dispersal-
470 mediated effects on populations in a highly context-dependent manner.
471 At a community level, maternal programming may impact ecosystem dynamics
472 by changing energy and material flow within and between trophic levels (Hawlena and
473 Schmitz 2010). Elevated maternal GCs result in greater offspring GCs, which affects
474 metabolic rate and digestive processes, and increases gluconeogenesis (Wingfield et al.
475 1998; Sapolsky et al. 2000). Higher metabolism leads to greater energy expenditure at
476 rest and animals will compensate for higher maintenance costs by increasing foraging
477 quantity or by foraging on higher quality prey. This is exacerbated by the fact that
478 increased GCs reduce digestive efficiency and energy intake, thus reducing conversion
479 efficiency of assimilated nutrients into body tissues. Pre-programmed offspring with
480 higher GC levels also have greater gluconeogenesis. Greater gluconeogenesis leads to
481 increased breakdown of proteins to produce glucose and can substantially change
482 body-nutrient composition, reducing N-rich proteins (Sterner and Elser 2002).
483 Gluconeogenesis may also increase N-excretion and, because proteins (amino acids)
484 are the major N-containing molecule, this will increase body C:N ratio (Sterner and Elser
485 2002). Thus, offspring will have reduced energy stores to fuel greater energy demands
486 and likely forage preferentially on higher quality prey. However, they may also have an
487 altered body-nutrient composition leading to impaired growth, development and body
488 condition reducing competitive ability within a trophic level and their overall value to
489 upper level predators. With energy flow reduced by 90% between trophic levels even
490 small changes in energy flow through one trophic level may have severe consequences
491 for the ecosystem as a whole. One emerging generality is that maternal programming

has context dependent effects on offspring phenotypes that may have cascading effects at the ecosystem level.

As this review has noted, many parallels between ecological and laboratory data exist which can serve to foster both collaborations and inspiration for further integration based on the strengths inherent in each approach. It remains critical to test hypotheses about underlying molecular and epigenetic mechanisms derived from laboratory studies in natural populations, where the timing, intensity, and ecological relevance of manipulations early in life may have distinct consequences. For ecological studies of maternal stress programming to remain relevant, researchers must begin linking the proximate effects of maternal stress programming with offspring and maternal fitness.

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Figure Legend

Figure 1. The hypothalamic-pituitary-adrenal (HPA) axis and negative feedback response of Glucocorticoids (GCs). The sensitivity of the feedback response is due to the level of GCs and the number of GC and mineralcorticoid receptors (GR and MR, respectively) in the brain and GR in the body (De Kloet et al. 1998; Wingfield and Sapolsky 2003; Seckl 2004). High levels of maternal stress during gestation or altered maternal care (due to high levels of maternal stress) shortly after birth can program the offspring brain, decreasing the number of receptors, reducing the feedback sensitivity and ultimately increasing offspring GC levels and associated behaviors (boxes; Welberg and Seckl 2001; Weaver et al. 2004; Abe et al. 2007; Meaney et al. 2007; Emack et al. 2008; Sheriff et al. 2010).

Figure 2. (A) DNA methylation of glucocorticoid receptor (GR) promoter regions occurs in offspring of low licking and grooming mothers (decreased maternal care associated with high GC levels). High levels of DNA methylation of this promoter prevent transcription factor (NGFI-A) binding and greatly reduce GR expression. (B) However, in offspring of high licking and grooming mothers (increased maternal care associated with low GC levels) the GR promoter region shows lower levels of DNA methylation, associated with enhanced GR expression (Weaver et al. 2004, 2005, 2007;McGowan et al., 2011).

Figure 3. Fecal cortisol metabolite (FCM) concentration (means ± SE) in free-ranging snowshoe hare dams and juveniles ($r^2 = 0.73$, $P = 0.007$). Each point is the average from a different litter group (1-3) in 2005- 2008. Juveniles were sampled within one week of weaning, 28 days after dams (i.e., juveniles are facing different conditions at the time of sampling than dams). Inset shows how juvenile FCM levels at weaning mirror that of dams at the time they gave birth (adapted from Sheriff et al. 2010).

913 Figure 4. Relative responsiveness of the stress axis in free-living European starling
914 fledglings in relation to natural variation in exposure to maternal stress (changes in yolk
915 corticosterone across laying order; see Love et al. 2008) and an experimental increase
916 in maternal stress (CORT-injections of eggs); adapted from Love and Williams (in prep).
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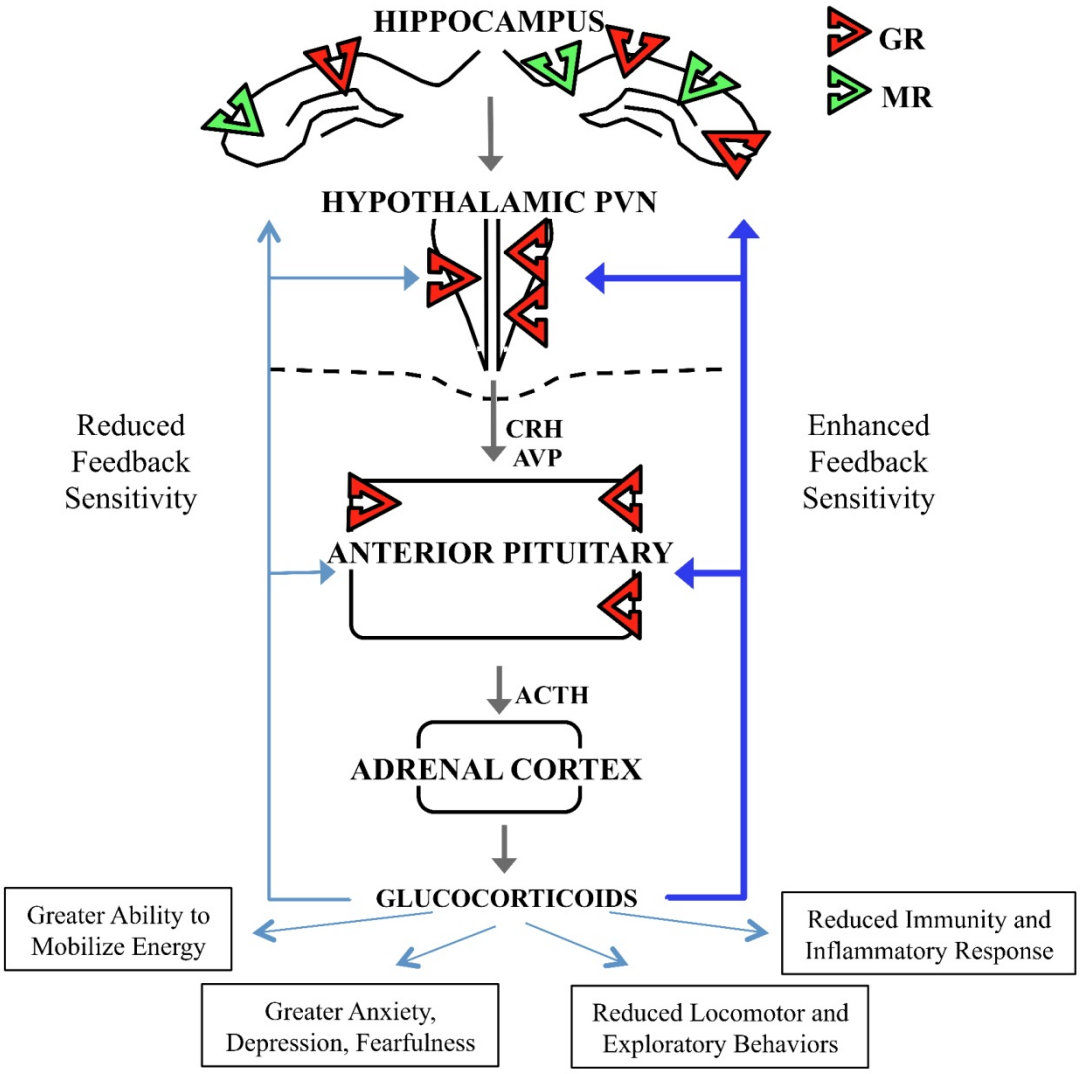
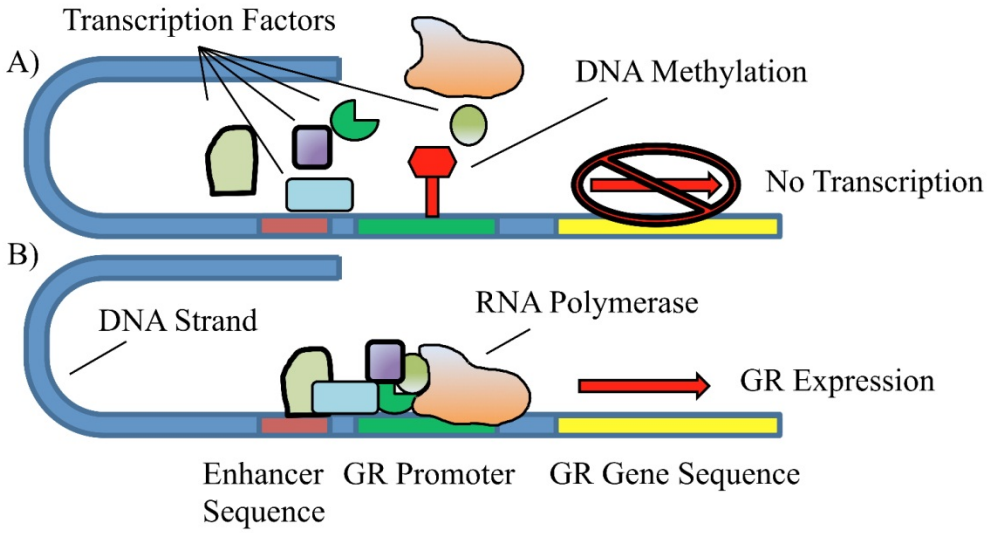
919 **Figure 1.**



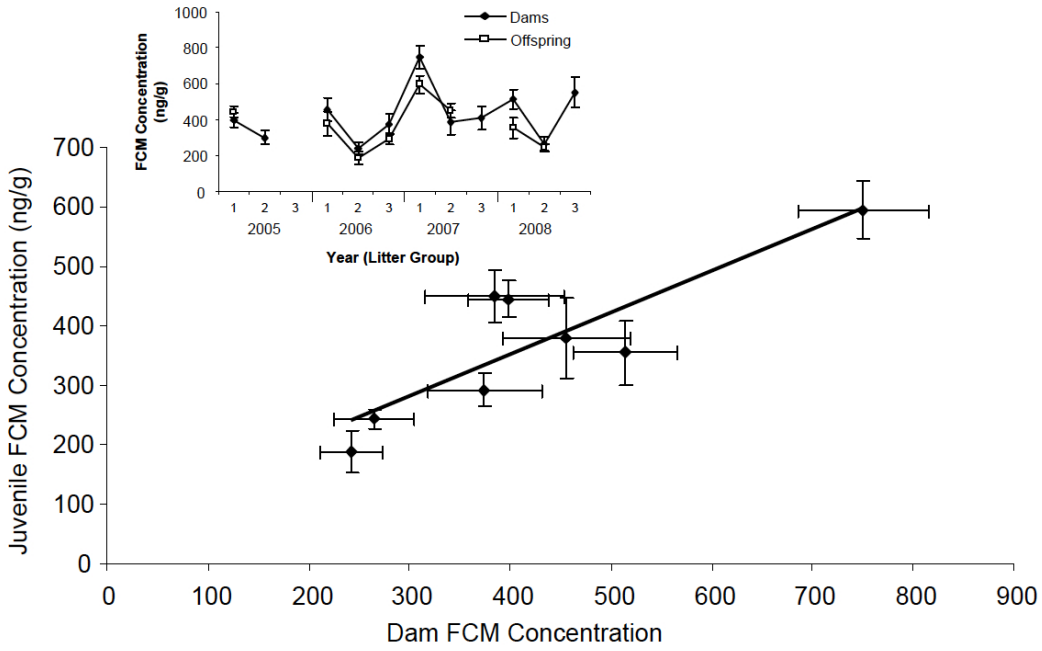
920

Figure 2.


Transcription Factors
A)
DNA Methylation
No Transcription
B)
DNA Strand
RNA Polymerase
GR Expression
Enhancer Sequence
GR Promoter
GR Gene Sequence

925

926 **Figure 3.**



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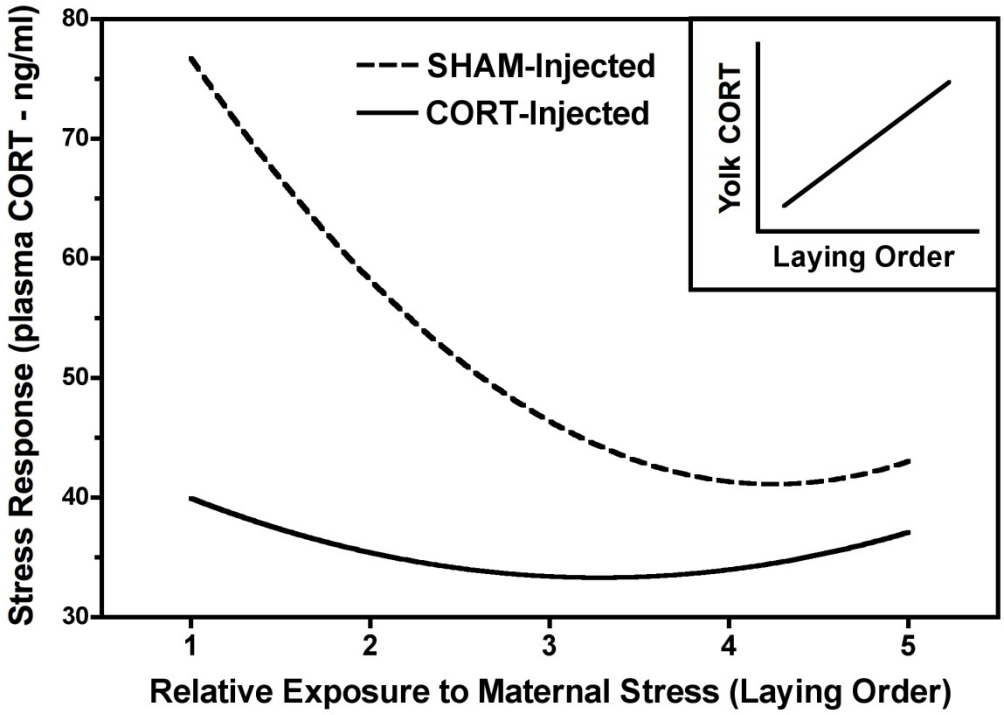
929 **Figure 4.**



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The neurological ecology of fear: insights neuroscientists and ecologists have to offer one another

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That the fear and stress of life-threatening experiences can leave an indelible trace on the brain is most clearly exemplified by post-traumatic stress disorder (PTSD). Many researchers studying the animal model of PTSD have adopted utilizing exposure to a predator as a life-threatening psychological stressor, to emulate the experience in humans, and the resulting body of literature has demonstrated numerous long-lasting neurological effects paralleling those in PTSD patients. Even though much more extreme, predator-induced fear and stress in animals in the wild was, until the 1990s, not thought to have any lasting effects, whereas recent experiments have demonstrated that the effects on free-living animals are sufficiently long-lasting to even affect reproduction, though the lasting neurological effects remain unexplored. We suggest neuroscientists and ecologists both have much to gain from collaborating in studying the neurological effects of predator-induced fear and stress in animals in the wild. We outline the approaches taken in the lab that appear most readily translatable to the field, and detail the advantages that studying animals in the wild can offer researchers investigating the "predator model of PTSD."

Keywords: animal model of PTSD, indirect predator effects, post-traumatic stress disorder, predation risk, predator stress

INTRODUCTION

Post-traumatic stress disorder (PTSD) represents arguably the most salient example of how fear and stress shape the mind. Because controlled prospective studies cannot be conducted on humans, it is necessary to use an "animal model" to help elucidate the etiology of PTSD and explore the associated neurological changes (Cohen et al., 2010). A suitable animal model should utilize stressors that emulate as closely as possible the relevant stressors in humans; the behavioral, physiological, and neurological responses elicited in the animal must reflect clinical symptomatology; and pharmacological agents known to affect symptoms in human patients should correct, with equal efficacy, comparable symptoms in the animal (Rosen and Schulkin, 1998; Roseboom et al., 2007; Stam, 2007; Armario et al., 2008; Masini et al., 2009; Mitra et al., 2009; Cohen et al., 2010).

Many researchers have adopted utilizing exposure to a predator (e.g., showing a rat a cat; Adamec and Shallow, 1993), or predator odor, as a stressor, in exploring the animal model of PTSD (Cohen et al., 2010; Mackenzie et al., 2010). Predator exposure was initially seized upon for practical reasons as this permits the researcher to utilize a (1) psychological stressor, that is (2) life-threatening, but (3) does not involve pain; all consistent with the etiology of PTSD in humans (Adamec and Shallow, 1993; Roseboom et al., 2007; Campeau et al., 2008; Takahashi et al., 2008; Staples et al., 2009; Cohen et al., 2010; Mackenzie et al., 2010). Of greatest importance with respect to understanding PTSD, the hallmark of which is the long-lasting or "transformational" change in the patient in response to a trauma (Yehuda and Bierer, 2009), predator exposure has been demonstrated to have long-lasting effects on: anxiety-like behaviors, glucocorticoid levels, dendritic morphology, gene expression, and the release of the neuropeptide corticotrophin-releasing hormone (CRH) in the amygdala (the region of the brain most frequently linked to fear), as well as many other phenomena associated with PTSD (Adamec and Shallow, 1993; Schulkin et al., 2005; Roseboom et al., 2007; Stam, 2007; Armario et al., 2008; Campeau et al., 2008; Rosen et al., 2008; Takahashi et al., 2008; Masini et al., 2009; Mitra et al., 2009; Staples et al., 2009). Predator exposure early in life has also been shown to increase vulnerability to developing subsequent long-term behavioral disruptions when exposed to a predator in adulthood (Cohen et al., 2006), consistent with the growing evidence that individual variation in susceptibility to PTSD is influenced by early-life experiences (Yehuda and Bierer, 2009).

Researchers studying the "predator model of PTSD" have increasingly begun to suggest that predator exposure offers an additional advantage in attempting to understand PTSD, because long-lasting predator-induced fear and stress is ethologically and ecologically relevant, and represents a valid experience applicable to animals in their natural environment (Roseboom et al., 2007; Cantor, 2009; Staples et al., 2009; Cohen et al., 2010). Independently, wildlife ecologists have begun to arrive at a similar conclusion, following a line of inquiry that began in the 1990s (Creel and Christianson, 2008). Traditionally, the view of both wildlife ecologists and comparative endocrinologists has been that

predator-induced fear and stress is necessarily acute and transitory: the prey detects a predator; freezes, flees, or fights; survives or does not; the event is over; the animal returns to going about its business; homeostasis is restored (Schulkin, 2003; Sheriff et al., 2009). According to this traditional view, lasting effects are necessarily maladaptive and pathological: since the evolutionary "function" of predator-induced fear and stress is to ensure immediate survival, any further or lasting effect on fitness (i.e., Darwinian fitness), such as an effect on subsequent reproduction, must be maladaptive; and since the "function" of the stress axis is to maintain homeostasis, chronic stress must be pathological (Lupien et al., 2009; Rodrigues et al., 2009; Sheriff et al., 2009). Given this traditional view, the many lasting effects of predator exposure documented by researchers exploring the predator model of PTSD must be an artifact. The most parsimonious explanation being – given this perspective – that such lasting effects stem from the unnatural conditions of captivity, i.e., it is not the fact of predator exposure but the fact the predator is inescapable that must explain these effects, since the animal cannot flee from the predator as it naturally would (Creel et al., 2009; Jöngren et al., 2010).

We propose that the traditional view in wildlife ecology and comparative endocrinology, that the effects of predators on free-living animals are necessarily transitory, is no longer tenable, since the results from a growing number of experimental and observational field studies show that predator-induced fear and stress has long-lasting effects on animals in the wild (Creel and Christianson, 2008; Hawlena and Schmitz, 2010), comparable to those documented by investigators addressing the predator model of PTSD. For animals in the wild that are in peril every moment of every day of being torn limb from limb by any number of predators, responses resembling PTSD in humans may result from necessary trade-offs to stay alive, that are fully adaptive, because dead animals do not reproduce. We suggest that for both, researchers studying the predator model of PTSD, and ecologists, conducting collaborative studies on predator-induced fear and stress on animals in the wild would be of enormous benefit. For investigators addressing the predator model of PTSD, the extremity of the stressors faced by animals in the wild, in a real world context, would appear to much better emulate the circumstances leading to PTSD in humans. For ecologists, building upon the progress that has been made in understanding PTSD in the lab provides the most expedient means of addressing the mechanisms underlying predator-induced fear and stress effects in the field. We briefly review approaches taken to studying PTSD in the lab that appear translatable to the field; and then describe recent field studies on songbirds and snowshoe hares showing that, predator-induced fear and stress affects reproduction in animals in the wild, and the physiological responses involved appear comparable to those documented in response to predator exposure in the lab.

APPROACHES TRANSLATABLE TO THE FIELD

Behavioral responses to predator exposure in the lab include avoidance, reduced activity and increased vigilance (Blanchard and Blanchard, 1989; Stam, 2007; Armario et al., 2008; Takahashi et al., 2008), and similar responses to predator exposure have been exhaustively documented in the field since at least Darwin's time (Caro, 2005). Predator exposure in the lab results in changes in plasma glucocorticoid levels (Blanchard et al., 1998; Roseboom et al., 2007; Takahashi et al., 2008; Masini et al., 2009) and the same has been shown in both birds and mammals in the field (Hawlena and Schmitz, 2010). Measuring glucocorticoid metabolites in feces provides a new, non-invasive means of assessing glucocorticoid responses to predator exposure that is particularly useful in field studies (Sheriff et al., 2009, 2010).

Studying the neurological effects of predator-induced fear and stress in animals in the wild will likely rely primarily on destructive sampling. Though effects on live animals could be studied using pharmacological methods or neuroimaging, there are practical difficulties translating these approaches to the field. The suitability of using predator exposure in exploring the animal model of PTSD has been validated, in part, by the numerous studies showing that pharmacological agents known to affect symptoms of PTSD in human patients also correct comparable symptoms in animals exposed to predators (Cohen et al., 2006, 2010; Stam, 2007; Armario et al., 2008; Nanda et al., 2008). Some of these pharmacological agents can be administered in food (e.g., antalarmin; Zoumakis et al., 2006; Armario et al., 2008), which is of practical advantage for use with free-living animals since it is then not necessary to capture the subject to administer the drug. The principal constraint on using pharmacological agents on animals in the wild is almost certain to be the cost of the drugs, since the intrinsically greater error variation associated with studying any phenomenon in the field necessitates a larger sample size than that required in the lab.

A number of recent neuroimaging studies using magnetic resonance imaging (MRI) have evaluated the neurological effects of exposure to predator odor in lab rats (e.g., Chen et al., 2007; Febo et al., 2009; Huang et al., 2011). MRI has also been used to assess neuroactivity in response to other stimuli in mice and songbirds (Van der Linden et al., 2007). Neuroimaging holds enormous promise as a technique for studying effects on animals in the wild because, being non-destructive, subjects could be returned to the field to determine if differences in brain activity predicted their subsequent behavior and reproduction. However, though MRI is non-destructive it is necessarily invasive and may be very injurious depending upon the method used (e.g., the manganese used in manganese-enhanced MRI is potentially toxic; Silva et al., 2004). At a minimum, neuroimaging requires restraining the subject's head in a scanner for a protracted period. To measure effects in conscious animals requires acclimation to being restrained in this manner, which takes several days in laboratory animals (King et al., 2005), and may be unachievable in many wild-caught animals. Even if anesthetized during the procedure, the trauma of capturing a wild animal and transporting it to wherever the scanner is might render the results uninterpretable (Van der Linden et al., 2007). Nonetheless, we strongly recommend that using neuroimaging to study effects on animals in the wild should at least be attempted.

Because animals in the wild are generally challenging to capture, and limited in number, it is critical to maximize the information extracted from every animal euthanized. Moreover, because free-living animals must be captured, the conditions of capture will vary, meaning the rate at which tissue can be obtained will vary, and the circumstances will often be less than ideal. Measures that respond to an acute trauma or perturbation, such as the trauma of capture, will be largely unsuitable. Several new approaches to

measuring neurological effects, developed in the lab, nonetheless appear amenable to use on animals in the wild, even given these constraints.

Immunohistochemistry has been used to map the expression of genes in response to predator exposure in various brain regions that appear central to the phenomenon of fear (such as the medial amygdala). Whereas a number of lab studies have mapped the expression of the immediate-early gene c-*fos*, in response to predator exposure (Dielenberg et al., 2001; Roseboom et al., 2007; Campeau et al., 2008), c-*fos* is rapidly expressed (within <1 h; Armario et al., 2008) and rapidly down-regulated (Staples et al., 2009), which is problematic for use in the field. Two recent studies (Staples et al., 2009; Mackenzie et al., 2010) have mapped the expression of *fos*B and its protein products FosB/ΔFosB, as an alternative to mapping c-*fos*. ΔFosB can persist in the brain for weeks after chronic stimulus exposure (McClung et al., 2004), and Staples et al. (2009) reported that FosB/ΔFosB expression remained elevated 7 days after repeated predator exposure, making this a much more suitable marker for use in field studies.

Global gene expression has been assessed in response to predator exposure using cDNA microarrays (gene chips) in rats and chickens. Roseboom et al. (2007) euthanized rats 3 h after predator exposure, and found increased CRH-binding protein gene expression in the amygdala, consistent with previous studies (Schulkin et al., 2005). Jöngren et al. (2010) euthanized chickens 2 week after predator exposure and identified 13 significantly differentially expressed genes in the midbrain. Roseboom et al.'s (2007) findings confirm that cDNA microarrays can be used to identify the expression of genes expected to be upregulated in response to fear, and Jöngren et al.'s (2010) study shows that this approach can be used to detect long-lasting effects, even in non-mammalian subjects.

Quantifying dendritic morphology appears ideally suited for indentifying individual variation in susceptibility to predator-induced fear and stress in field studies, and may be useful in evaluating predator-induced changes in neural architecture. Mitra et al. (2009) evaluated behavioral differences in subjects 2 weeks after predator exposure and found differences in the architecture of the neurons in the basolateral amygdala. Total dendritic length, dendritic extent, and total branch points were all greater in individuals that continued to demonstrate anxiety-like behaviors as compared to those that no longer showed anxiety-like symptoms. Though the design of Mitra et al.'s (2009) study did not allow them to determine whether these differences in dendritic morphology were pre-existing or induced by predator exposure, Mitra and Sapolsky (2008) reported changes in dendritic morphology in response to a single day of stress, suggesting that predator-induced fear could indeed induce such changes in neural architecture.

Yehuda and Bierer (2009) recently reviewed the potential role of epigenetic changes in the etiology of individual differences in susceptibility to PTSD. Epigenetic modifications involve long-lasting, often environmentally induced, changes in gene expression and function, that can be inter-generationally transmissible (i.e., heritable), though the DNA sequence itself remains unchanged (Champagne and Curley, 2009; Yehuda and Bierer, 2009). Several lines of evidence point to epigenetic changes as potentially being involved in predisposing individuals to PTSD, including the association of PTSD risk with maternal PTSD, the relevance of childhood adversity to the development of PTSD, and recent evidence of a relationship between childhood abuse, DNA methylation (in gene promoters, an epigenetic marker of gene silencing) and suicide (McGowan et al., 2008, 2009; Yehuda and Bierer, 2009). As noted above, Cohen et al. (2006) reported that early-life predator exposure increased vulnerability to behavioral disruptions in response to exposure in adulthood, though there have been no studies looking specifically at predator-induced epigenetic changes. In the aforementioned suicide study, subjects had been dead an average of 24 h before sampling, suggesting that changes in DNA methylation ought to be detectable in the brains of animals in the wild collected under less than ideal field conditions, as recently corroborated by Pilsner et al. (2010) in a study that examined DNA methylation in the brains of polar bears shot by aboriginal hunters in eastern Greenland.

FIELD STUDIES SHOWING LONG-LASTING EFFECTS OF PREDATOR EXPOSURE

Evolutionarily, the "function" of staying alive is to reproduce, i.e., to transmit genes to the next generation. For ecologists, reproduction is the "currency" that matters. Ecological factors such as food and parasites, with obvious long-lasting effects (malnutrition and disease), have always been considered to be those most likely to affect reproduction, because reproduction (giving birth and rearing young) is a slow process. Traditionally, predators have not been thought to affect reproduction because predator-induced fear and stress has been considered to be far too acute and transitory. Behavioral (e.g., Kotler, 1992) and physiological (e.g., Boonstra et al., 1998) studies began, in the 1990s, to suggest that predator-induced fear and stress could have lasting effects on animals in the wild, but because of the logistical challenges involved the critical experiments necessary to demonstrate effects on reproduction have only very recently been conducted. The principal challenge concerns space. Free-living animals can, and do, simply flee or avoid, a predator in a cage, predator models, or predator odor stations (e.g., Stankowich and Blumstein, 2005). Because sound travels, and thus occupies space, field studies often use playbacks of recorded predator calls and sounds to investigate effects of predator exposure. Moreover, for organisms that rely more on sound and sight than smell, such as birds and humans, auditory stimuli are generally more meaningful than olfactory ones (Jarvis, 2004), and acoustic cues may frequently be more alarming than visual ones (Cohen et al., 2010).

Only one study to date on a bird or a mammal has, to our knowledge, exposed free-living prey to increased predator cues in the field, and demonstrated a resulting effect on the number of offspring produced per year. Zanette et al. (submitted) used an array of speakers spaced over several hectares to expose nesting female song sparrows to playbacks of either predator calls and sounds, or non-threatening calls and sounds. Females exposed to elevated predation threat produced almost 40% fewer offspring than controls (3.8 ± 0.4 vs. 6.0 ± 0.4, mean $\pm$ SE), over the 4-month breeding season, because they laid fewer eggs, fewer of their eggs hatched, and more of their chicks starved to death. These effects on reproduction were most likely mediated in part by predator-threat-induced changes in glucocorticoid levels, because work on the same study populations has

demonstrated lasting effects on glucocorticoid levels associated with variation in predator abundance (Clinchy et al., 2004, 2011), and the probability of suffering nest predation (Travers et al., 2010).

Sheriff et al. (2009) recently reported correlative results suggesting that predator exposure affects glucocorticoid levels and reproduction in free-living snowshoe hares, consistent with the results from Zanette et al.'s (submitted) experiment. To corroborate their findings, Sheriff et al. (2009) presented a live predator (a trained dog) to pregnant hares housed in 4 m × 4 m outdoor pens, and demonstrated that predator-exposed females had dramatically elevated fecal glucocorticoid metabolite (FCM) levels, and were significantly less likely to give birth to live young.

In a subsequent study on snowshoe hares, Sheriff et al. (2010) showed that predator exposure may have very long-lasting effects on animals in the wild, extending from one generation to the next. Sheriff et al. (2010) reported that at a population level, predator exposure, mean maternal FCM levels, and mean juvenile FCM levels, were all correlated, suggesting that predator-induced glucocorticoid changes in mother hares affect their offspring's glucocorticoid levels. To corroborate these findings, Sheriff et al. (2010) measured FCM levels in pregnant hares, housed in 4 m × 4 m outdoor pens, and demonstrated that each mother's FCM level was highly correlated with her offspring's glucocorticoid responses to a hormonal challenge, when the latter was 28 days old. Thus, in animals in the wild, maternal or early-life exposure to predators may increase responsiveness to predators later in life, consistent with Cohen et al.'s (2006) lab results demonstrating that early-life predator exposure increases vulnerability to behavioral disruptions when exposed to a predator in adulthood.

Life-long maternal effects on the glucocorticoid responsiveness of their offspring, resembling the results shown by Sheriff et al. (2010), have been well-studied in relation to stress effects on maternal care in laboratory rodents, and have been shown to be associated with DNA methylation of genes affecting glucocorticoid receptor function in the hippocampus (Weaver et al., 2004; Kappeler and Meaney, 2010). In an example of the kind of collaboration between neuroscientists and ecologists we are herein hoping to encourage, McGowan and Boonstra are currently examining the brains of juvenile snowshoe hares, collected in the field, whose mothers were subject to naturally varying levels of predator exposure, to test if maternal predator exposure affects DNA methylation in their offspring's hippocampus in a manner similar to the way in which childhood abuse evidently affects DNA methylation in humans, as shown in the aforementioned suicide study (McGowan et al., 2008, 2009).

Calisi and Bentley (2009) recently proposed that studying neurobiology and behavior in semi-natural settings may provide a means to merge lab and field approaches. Our focus here is on the lasting effects of predator-induced fear and stress on neurobiology and ecology. As noted above, the principal challenge in studying such lasting ecological effects concerns space, and this applies equally to studying such effects in a semi-natural setting – the subject must have the same amount of space available as it would if it were free-living, to flee or avoid a predator, otherwise any effects seen could be attributed to the unnatural conditions of captivity (Creel et al., 2009). Sheriff et al.'s (2009, 2010) exposure of caged hares to a predator, for example, cannot be considered definitive, for this reason (Clinchy et al., 2011). Moreover, since, as noted above, the ecological "currency" that matters is reproduction, the subject must be able to reproduce as it naturally would. Very large (e.g., several hundred square meter) outdoor enclosures may fulfill these requirements when studying very small animals (e.g., mice or songbirds), whereas housing an animal in a somewhat larger cage than usual in an animal care building (e.g., Blanchard and Blanchard, 1989; Choi and Kim, 2010) does not meet these criteria.

The scope for future collaborations between neuroscientists and ecologists will almost certainly involve studying many more species than just sparrows and hares. As further field experiments on the effects of predator exposure on reproduction are conducted, we have no doubt such effects will be found to be common. Effects on components of reproductive success have already been documented in experiments on several other species. Eggers et al. (2006) reported effects of predator call playbacks on the number of eggs laid by Siberian jays, and Fontaine and Martin (2006) found that where predators were removed songbirds laid heavier eggs. Karels et al. (2000) similarly showed that where predators were removed the proportion of arctic ground squirrel females weaning young was increased. Lasting behavioral and physiological effects pointing to likely effects on reproduction have been shown in an even larger number of species (Creel and Christianson, 2008; Hawlena and Schmitz, 2010). What effect such predator-induced fear and stress has in shaping the minds of free-living birds and mammals is a question that is almost completely unexplored, presenting a wide-open field of study replete with opportunities for new discoveries.

CONCLUSION

Numerous laboratory experiments have shown that predator-induced fear and stress has lasting neurological effects, and wildlife ecologists have begun demonstrating that predator-induced fear and stress has lasting effects on reproduction in free-living animals in the field. We propose that the next two critical questions to answer are: (1) whether predator-induced fear and stress has lasting neurological effects on free-living animals, and if so; (2) which of the effects seen in the lab appear most frequently in wild animals in the field. The insights neuroscientists have to offer ecologists in exploring the effects of predator-induced fear and stress on the minds of wild animals in the field include, where to begin, and what to measure. The insights ecologists, in turn, have to offer researchers studying the predator model of PTSD include, establishing which effects seen in the lab are observed in the greatest number of species and circumstances, and which are most biologically meaningful as gaged by their association with effects on reproduction. We suggest that if, as the predator model assumes, PTSD in humans has evolutionary precursors, then it is virtually certain that collaborations between neuroscientists and ecologists will greatly enhance our understanding of the etiology of PTSD and the associated neurological changes.

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PLoS one

Broad Epigenetic Signature of Maternal Care in the Brain of Adult Rats

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Abstract

Background: Maternal care is associated with long-term effects on behavior and epigenetic programming of the *NR3C1* (*GLUCOCORTICOID RECEPTOR*) gene in the hippocampus of both rats and humans. In the rat, these effects are reversed by cross-fostering, demonstrating that they are defined by epigenetic rather than genetic processes. However, epigenetic changes at a single gene promoter are unlikely to account for the range of outcomes and the persistent change in expression of hundreds of additional genes in adult rats in response to differences in maternal care.

Methodology/Principal Findings: We examine here using high-density oligonucleotide array the state of DNA methylation, histone acetylation and gene expression in a 7 million base pair region of chromosome 18 containing the *NR3C1* gene in the hippocampus of adult rats. Natural variations in maternal care are associated with coordinate epigenetic changes spanning over a hundred kilobase pairs. The adult offspring of high compared to low maternal care mothers show epigenetic changes in promoters, exons, and gene ends associated with higher transcriptional activity across many genes within the locus examined. Other genes in this region remain unchanged, indicating a clustered yet specific and patterned response. Interestingly, the chromosomal region containing the *protocadherin-α, -β,* and *-γ* (*Pcdh*) gene families implicated in synaptogenesis show the highest differential response to maternal care.

Conclusions/Significance: The results suggest for the first time that the epigenetic response to maternal care is coordinated in clusters across broad genomic areas. The data indicate that the epigenetic response to maternal care involves not only single candidate gene promoters but includes transcriptional and intragenic sequences, as well as those residing distantly from transcription start sites. These epigenetic and transcriptional profiles constitute the first tiling microarray data set exploring the relationship between epigenetic modifications and RNA expression in both protein coding and non-coding regions across a chromosomal locus in the mammalian brain.

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Introduction

The quality of parental care has a broad impact on mental health, including the risk for psychopathology [1,2,3,4,5]. Studies in the rat directly link the maternal care environment to long-term effects on neural systems that regulate stress [6,7] emotional function[8,9], learning and memory [10,11,12] and neuroplasticity [10,13,14,15]. Naturally occurring variations in maternal care in the first week of life in rats are associated with changes in brain and behavior that persist until adulthood [16]. These effects are reversed by cross-fostering, [7,9] demonstrating a causal link between maternal care and gene expression programming.

In rats and humans, there is evidence that changes in gene expression as a function of early care are at least partly regulated by epigenetic mechanisms [6,17,18]. In rats, variations in maternal care in the first week of life are associated with alterations in DNA methylation and H3K9 acetylation of the *NR3C1* promoter region, and gene expression of the $GR1_7$ splice variant of the *NR3C1* gene in the hippocampus of adult offspring [6]. There is evidence that the expression of hundreds of additional genes in adult rats changes in response to differences in maternal care [19]. Some of these changes in gene expression can be reversed by pharmacological alterations of chromatin structure by the histone deacetylase inhibitor Trichostatin A (TSA) and the methyl donor L-methionine [19,20]. The fact that the methyl donor L-methionine inhibits some of the genes influenced by maternal behavior supports the involvement of either DNA or histone methylation. The fact that a large number of genes are responsive to the effects

of TSA and L-methionine implies that the epigenetic regulation of gene expression as a function of maternal care may be extensive. In the present study, we test this hypothesis by examining epigenetic and transcriptional changes associated with naturally occurring differences in maternal care.

We obtained hippocampal samples from the adult offspring of rat mothers that differed in the frequency of pup licking/grooming in the first week of life (i.e. High vs Low LG adult offspring) and performed an analysis of DNA methylation, H3K9 acetylation and gene expression of a contiguous 7 million base pair region of rat chromosome 18 containing the *NR3C1* gene at 100 bp spacing. To our knowledge, these epigenetic and transcriptional profiles constitute the first tiling microarray data set exploring the relationship between epigenetic modifications and RNA expression in both protein coding and non-coding regions across a chromosomal locus in the mammalian brain.

Results

Validation of microarray results

To validate signals observed on our microarray and differences between High and Low LG offspring, we quantified changes in H3K9 acetylation, DNA methylation, and transcription. H3K9 acetylation differences in 7 regions (**Fig. 1a**) and DNA methylation differences in 12 regions (**Fig. 1b**) were validated by quantitative PCR (qChIP – see **Methods** for details; [21]). Levels of DNA methylation validated by qChIP correlated significantly with levels of enrichment detected by microarray ($R = 0.38$, $P = 0.0029$ by Pearson's correlation; **Fig. S1**). DNA methylation differences were further confirmed for four genes by sequencing sodium bisulfite converted DNA (**Fig. S2**). False positives due to DNA polymorphism rather than differential methylation were ruled out for 12 regions (those validated by qChIP above) via DNA sequencing (data not shown). Of nine genes showing significant differences in gene expression between High and Low LG offspring, all were significantly more expressed among High LG offspring (**Fig. 1c**).

As a further method of validating our microarrays, we compared our average observed levels of transcriptional and epigenetic signals to previously described signals within specific gene elements across the entire locus profiled. To do so, we examined the absolute levels of transcription, histone acetylation and DNA methylation for all subjects combined (**Fig. S3**), and compared them to previously published relationships between levels of gene expression, DNA methylation, and histone acetylation across 5′ regulatory regions, exons, and introns. First, previous studies have indicated that much of the genome is actively transcribed [22] but that levels of transcription are generally higher within annotated exons relative to other regions. As expected, inside exons we observed significantly higher transcription than the overall levels of transcription throughout all regions in the locus ($P = 1.47\times10^{-155}$ by Student's T-test, $P = 0$ by Wilcoxon Rank Sum test). In contrast, we observed levels of transcription just upstream of genes (−1800 bp to transcription start site) and in intronic regions that were indistinguishable from the baseline. These data indicate that the signals observed by our microarray accurately detect known transcribed regions. Second, many previous studies in a variety of cell types have shown that active transcription is associated with low levels of DNA methylation in the 5′ ends of genes [23]. CpG islands also show lower than average levels of DNA methylation compared to other genomic regions [24]. As expected, we observed lower DNA methylation levels in 5′ gene ends ($P = 1.34\times10^{-78}$ by Wilcoxon Rank Sum test) and within CpG islands ($P = 7.15\times10^{-200}$ by Wilcoxon Rank Sum test) than the overall levels of methylation across the locus (**Fig. S3b–c**). Third, actively transcribed genes have been associated with reduced nucleosome occupancy near transcription start sites [25,26,27]. We similarly found lower H3K9 acetylation levels in 5′ gene ends ($P = 9.22\times10^{-47}$ by Wilcoxon Rank Sum test; **Fig. S3b**). Computational prediction of nucleosome density from DNA sequence [28] showed a significant correlation between nucleosome position and H3K9 acetylation levels observed by microarray ($R = 0.2$, $P = 2.2\times10^{-16}$ by Pearson's correlation; **Fig. S4**). These observations of lower absolute levels of DNA methylation with CpG islands and higher levels of transcription within exons associated with lower DNA methylation and H3K9 acetylation levels in 5′ gene ends indicate that our epigenetic and transcription microarray results conform to previously published data in other genomic loci.

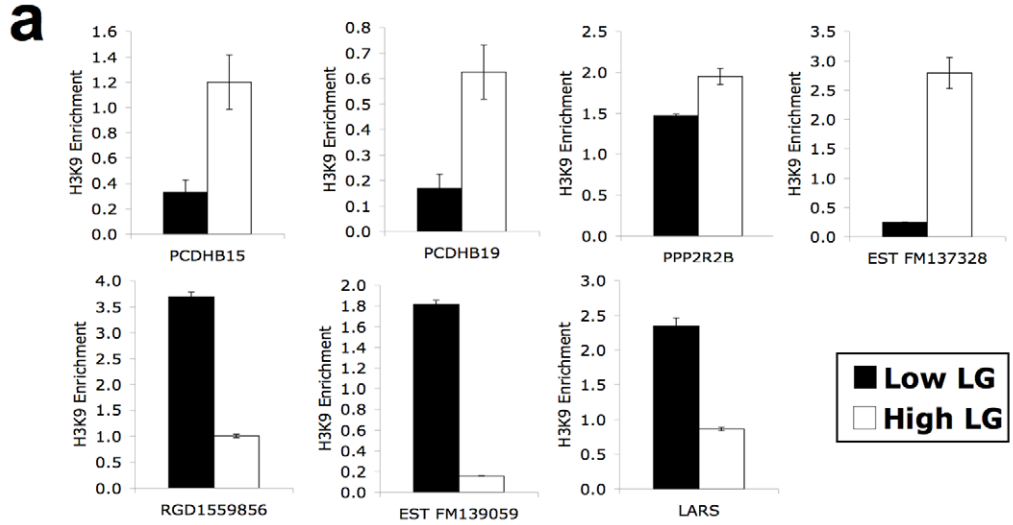
The pattern of the epigenetic and transcriptional response to maternal care across the *NR3C1* gene locus

A "large-scale" view of the entire locus as a whole revealed a widespread but patterned response to maternal care among High and Low LG adult offspring (High – Low; **Fig. 2**). We observed peaks and valleys of H3K9 acetylation and DNA methylation levels throughout a number of regions, suggesting a widespread epigenomic response to variations in maternal care. The response to maternal care is not evenly distributed, with many sequences showing little or no response and clustered regions showing enhanced responses. In total, we found significant differential DNA methylation in 1413 probes and significant differences in H3K9 acetylation in 713 probes out of 44000 probes covering the region. Variations in epigenetic signaling across the locus appear within annotated genic regions (e.g., **Fig. 2** – see blue highlight), and also in regions where no gene is annotated (e.g., **Fig. 2** – see orange highlight). Transcriptional differences are similarly widespread (**Fig. 2** – see expression track). These results suggest that some but not all regions are associated with changes in epigenetic signaling associated with differences in maternal care, with broad epigenetic changes apparent within both genic and inter-genic areas.

Localization of broad epigenetic changes to gene regulatory and transcriptional elements

To index broad epigenetic changes observed across the locus, we defined a Regional Difference in DNA methylation and a Regional Difference H3K9 acetylation (RDme and RDac, respectively) as a statistically significant difference between High LG and Low LG offspring of at least 1000 bp containing at least one statistically significant probe per 1000 bp (see **Methods S1** for details). Across the entire locus, we identified 723 RDme of which 373 are significantly hypermethylated and 350 are hypomethylated in High relative to Low LG offspring. We similarly identified 471 RDac of which 204 are hyperacetylated and 267 are hypoacetylated. We found that these broad epigenetic differences associated with maternal care are significantly co-localized within the locus, and were positively correlated at distances over 100 Kb (**Fig. 3a**). The data suggest that clustering of differentially methylated and acetylated regions is not exclusive to pathological responses under extreme selection as is the case in cancer but includes epigenetic responses to natural variations in maternal care, and may be characteristic of naturally occurring epigenetic responses.

We next examined the localization of broad differences in H3K9 acetylation, DNA methylation (i.e., RDac and RDme) and transcription with respect to the physical anatomy of genes within


a
H3K9 Enrichment
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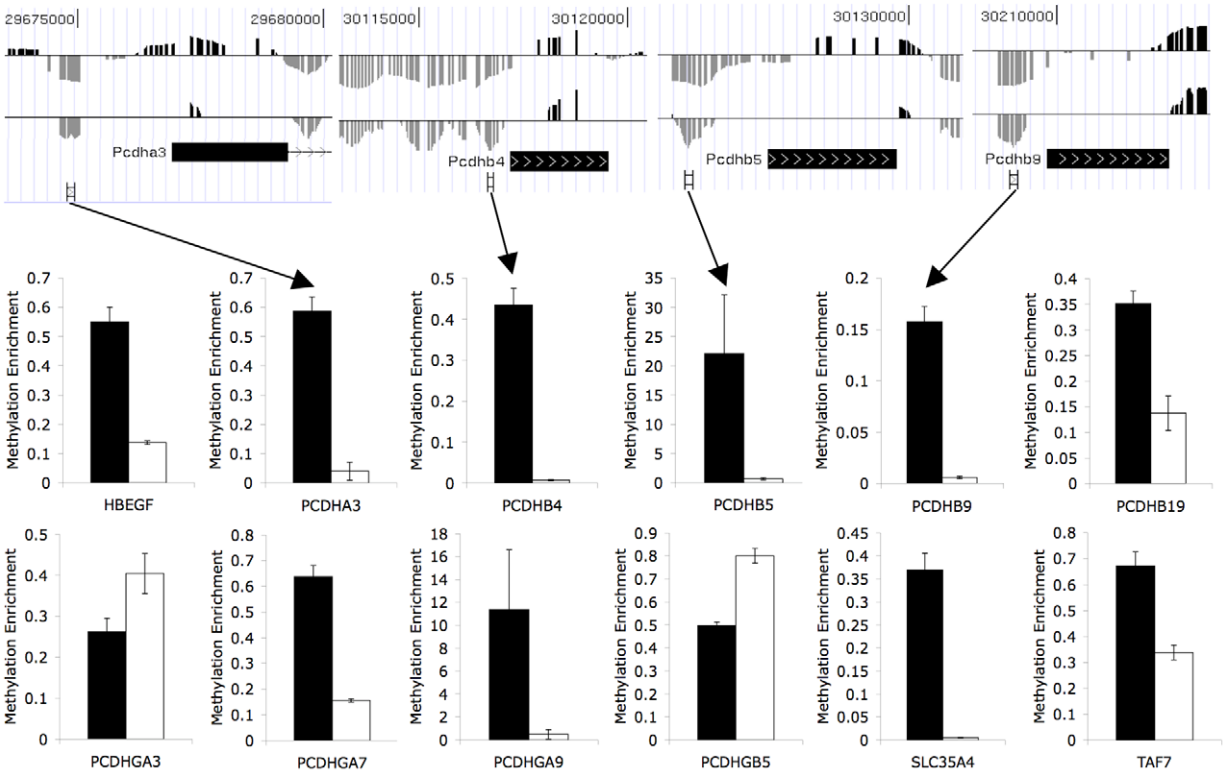
PCDHB19
PPP2R2B
EST FM137328
RGD1559856
EST FM139059
LARS
Low LG
High LG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PCDHGA3
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SLC35A4
TAF7

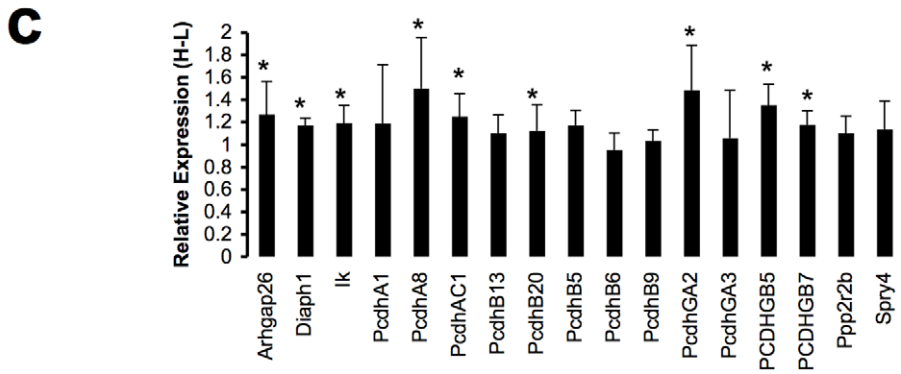
c
Relative Expression (H-L)
Arhgap26
Diaph1
Ik
PcdhA1
PcdhA8
PcdhAC1
PcdhB13
PcdhB20
PcdhB5
PcdhB6
PcdhB9
PcdhGA2
PcdhGA3
PCDHGB5
PCDHGB7
Ppp2r2b
Spry4

Figure 1. Microarray validation. (**a**) H3K9 acetylation differences between High (white bars) and Low LG (black bars) adult offspring validated by qCHIP (see **Methods**). (**b**) (upper) DNA methylation differences between High and Low LG adult offspring detected by microarray analysis (H–L), showing gene location, and region analyzed. (lower) DNA methylation differences validated in the same manner as for H3K9 acetylation. (**c**) Gene expression differences between High LG and Low LG adult offspring (* = $P<0.05$). All real-time PCR reactions were performed in triplicate and results are displayed as mean +/− SEM.
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the locus. Gene regulatory elements, including transcription start sites, 5′ and 3′ gene ends, and CpG islands are typical regions of interest in studies of gene regulation by epigenetic mechanisms. We found no evidence of a relationship between CpG density and the presence of RDme ($P=0.53$ by Wilcoxon rank sum test), indicating there is no difference between CpG islands and other regions with respect to the presence of RDme. RDme/ac overlapped the transcription start sites of some but not all genes, indicating specificity in epigenetic signaling within the locus. Seventy-seven transcription start sites in 69 genes contain RDme while 127 transcript start sites in 94 genes do not contain RDme. Similarly, 37 transcription start sites in 32 genes contain RDac while 167 transcription start sites in 131 genes do not contain RDac. There was a significant enrichment of hyperacetylated RDac (regions in which the high maternal care group has higher acetylation levels) inside exons, particularly the first and last exons ($P=0.0014$ and $P=0.0088$, respectively; permutation test), and a significant depletion of hypoacetylated RDac inside the first and last exons ($P=0.0002$ and $P=0.19$, respectively; permutation test). RDac are relatively depleted in the 5′ and 3′ ends of genes ($P=0.02$ by permutation test), likely reflecting the aforementioned depletion of nucleosomes at these sites in actively transcribed genes. In contrast, RDme co-localize in regulatory elements, particularly in the 5′ and 3′ ends of genes ($P=0.0032$ by permutation test). Hypermethylated RDme (regions that are more methylated in the high maternal group than in the low maternal care group) are significantly enriched inside both the first and last exons of genes ($P=0.0008$ and $P=0.004$, respectively; permutation test) whereas hypomethylated RDme are significantly depleted inside the first exon ($P=0.0022$; permutation test; **Fig. 3b** – red for hypermethylated RDme and blue for hypomethylated RDme). In addition, we observed an enrichment of hypomethylated RDme upstream of the TSS ($P=0.02$; **Fig. 3b** – blue line). These data showing an enrichment of hyperacetylated RDac and hypermethylated RDme within exons and an enrichment of hypomethylated RDme in regulatory elements are consistent with previous data in cancer cells showing high exonic H3K9 acetylation [25,26,27] and DNA methylation [29] and low promoter DNA methylation associated with actively transcribed genes.

Next, we performed an analysis of probe-level changes in epigenetic and transcriptional signaling as an alternative method to compare to previous studies in cancer. We compared probe-level differences in H3K9 acetylation, DNA methylation, and RNA transcription to (1) identify whether our data show a similar correspondence between higher levels of transcription observed in the High LG offspring and epigenetic changes we expect based on studies in cancer cells and (2) examine whether the observed patterns at the level of individual probes are indicative of our analyses of RDme and RDac. **Figure 3c** shows differences in H3K9 acetylation, DNA methylation, and RNA expression, with non-zero values indicating significant differences between High and Low LG offspring and line thickness denoting the standard error of the mean. In agreement with previous studies in cancer and the analyses of RDac/me above, H3K9 acetylation levels are significantly higher inside exons of the High LG offspring compared to the Low LG offspring, particularly the first and last exons (**Fig. 3c** – left panel). DNA methylation differences between the groups are, on average, significantly higher within exons among High LG offspring compared to Low LG offspring (**Fig. 3c** – middle panel). Expression differences inside exons indicate that High LG offspring show, on average, significantly higher RNA

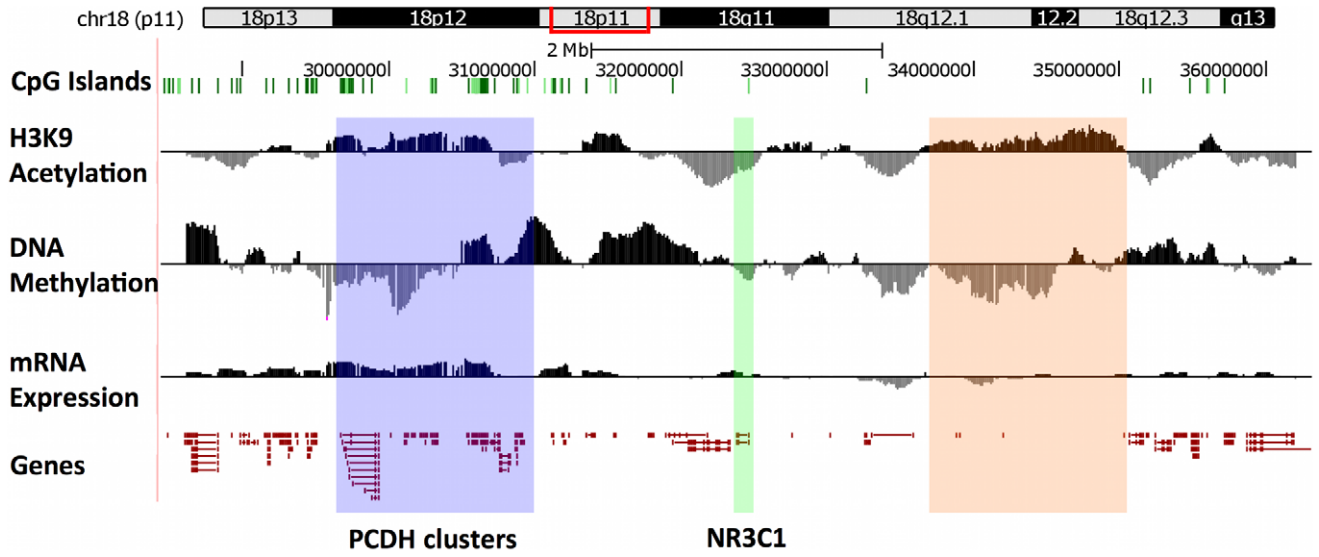

Figure 2. The pattern of H3K9 acetylation, DNA methylation, and gene expression among High and Low LG adult offspring across ~7MB of chromosome 18. Tracks show CpG Islands, differences in H3K9 acetylation, DNA methylation and gene expression between High (black) and Low LG (grey) adult offspring (H–L) and the locations of known genes (red) across the chromosomal locus (see **Methods S1**). Highlighted regions show the location of the *NR3C1* gene (green), Protocadherin gene clusters (blue) and a large mainly intergenic region (orange).
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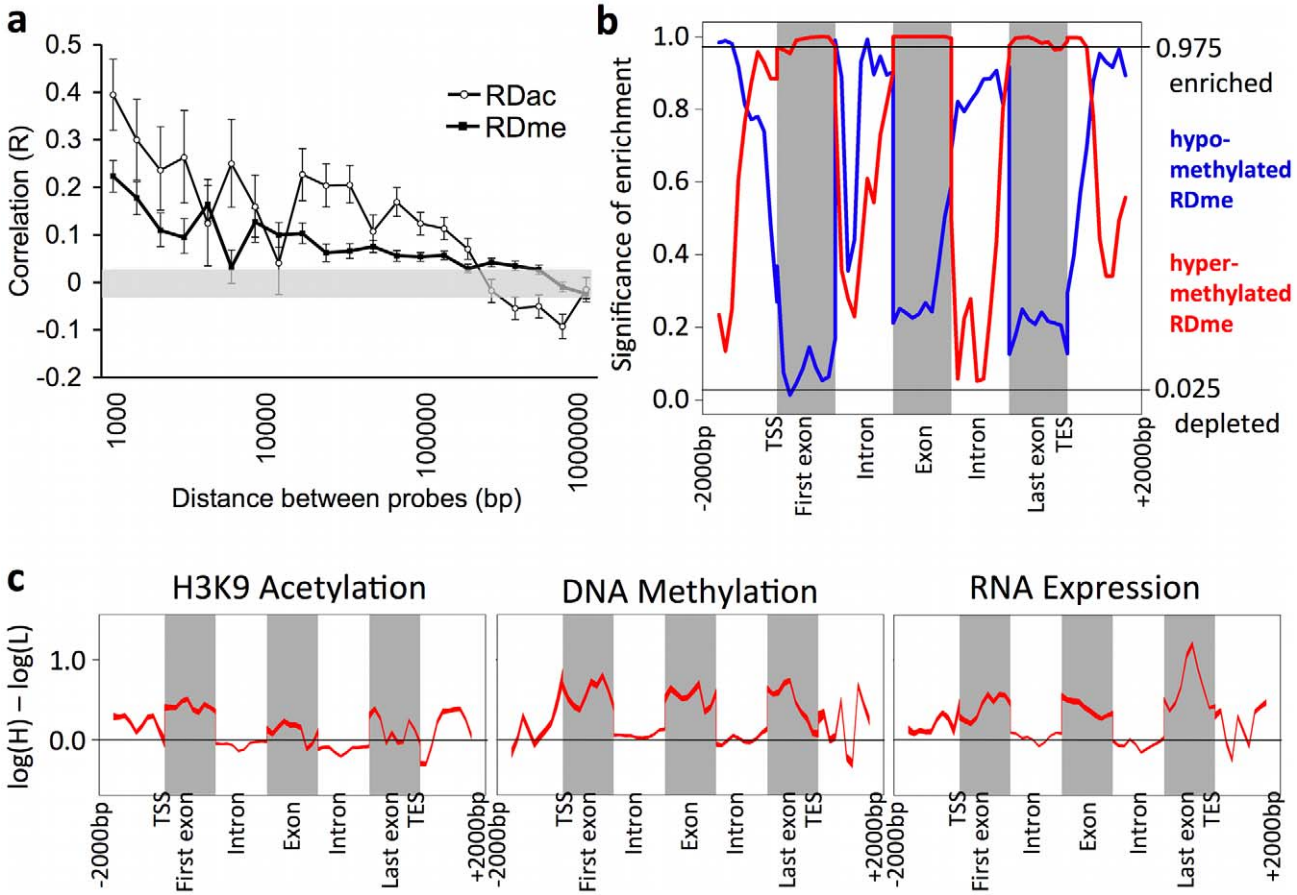

Figure 3. Regional variations in differences in histone acetylation, DNA methyation and gene expression between High and Low LG adult offspring. (**a**) The Pearson correlation of DNA methylation and H3K9 acetylation differences between the High and Low LG adult offspring for pairs of probes located at varying distances from each other. Error bars show 95% confidence intervals for the correlation values. Grey highlight shows the 95% confidence interval for correlations obtained from randomly selected probe pairs. (**b**) Enrichment of RDme (Regional Differences in DNA methylation) between High and Low LG adult offspring across all genes from the 5′ region to the 3′ region. Enrichment is quantified as increased frequency of RDme in a given gene region (number of RDme/bp). Significance is the quantile of this enrichment with respect to the distribution of randomly positioned RDme. A quantile above 0.975 indicates significant enrichment, and a quantile below 0.025 indicates significant depletion at the $P = 0.05$ level. Quantiles of hyperacetylated RDac/hypermethylated RDme in High compared to Low LG offspring (red) and quantiles of hypoacetylated RDac/hypomethylated RDme (blue) are shown. (**c**) Mean differences across all probes in DNA methylation, H3K9 acetylation and RNA expression levels between High LG and Low LG adult offspring are shown across all genes from the 5′ region to the 3′ region, with significant differences indicated by non-zero values. Line thickness denotes SEM.
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expression within annotated genes among High LG offspring compared to Low LG offspring (**Fig. 3c** – right panel). These data confirm previously published observations in cancer cells showing an association of actively transcribed genes with hyperacetylation and high methylation within exons [25,26,27,29]. Taken together, these analyses within the regulatory and transcriptional elements of the genes in the locus are consistent with an observed significantly higher overall transcriptional activity among High LG adult offspring.

NR3C1 gene and identification of novel candidate genes regulated by maternal care

We previously reported that NR3C1 gene expression and H3K9 acetylation were increased and DNA methylation was decreased in the promoter of the exon 1_7 splice variant among High LG offspring compared to Low LG offspring [6,7]. Using our comprehensive coverage of the entire NR3C1 locus we were able to identify additional novel regions of differential transcription, DNA methylation and histone acetylation in response to maternal care. We observed a number of RDme and RDac co-localized within intronic regions and upstream of the promoter region within the *NR3C1* gene (**Fig. 4a**). The *NR3C1* gene is known to contain at least 11 untranslated 5′ exon 1 splice variants that encode a common protein via a splice acceptor site on the exon 2. In this way, tissue-specific expression of NR3C1 is regulated by alternative splicing [30]. Our gene expression data agrees with previous studies showing that the expression of the GR exon 1_7 splice variant as well as that of exon 2 is increased in High LG offspring ([6,7]; **Fig. 4b**). Furthermore, we also detected increased transcription among High LG offspring in each of the exon 1 splice variants known to be expressed in the hippocampus: $GR1_5$, $GR1_6$, $GR1_7$, $GR1_{10}$, and $GR1_{11}$ ([30]; **Fig. 4b**). These results suggest that broad epigenetic differences within the NR3C1 gene as well as the coordinated expression pattern of NR3C1 splice variants may be involved in the response to maternal care.

In addition to the *NR3C1* gene, a number of other genes show a significant number of RDme and are induced in response to differences in maternal care. We observed a broad genomic region

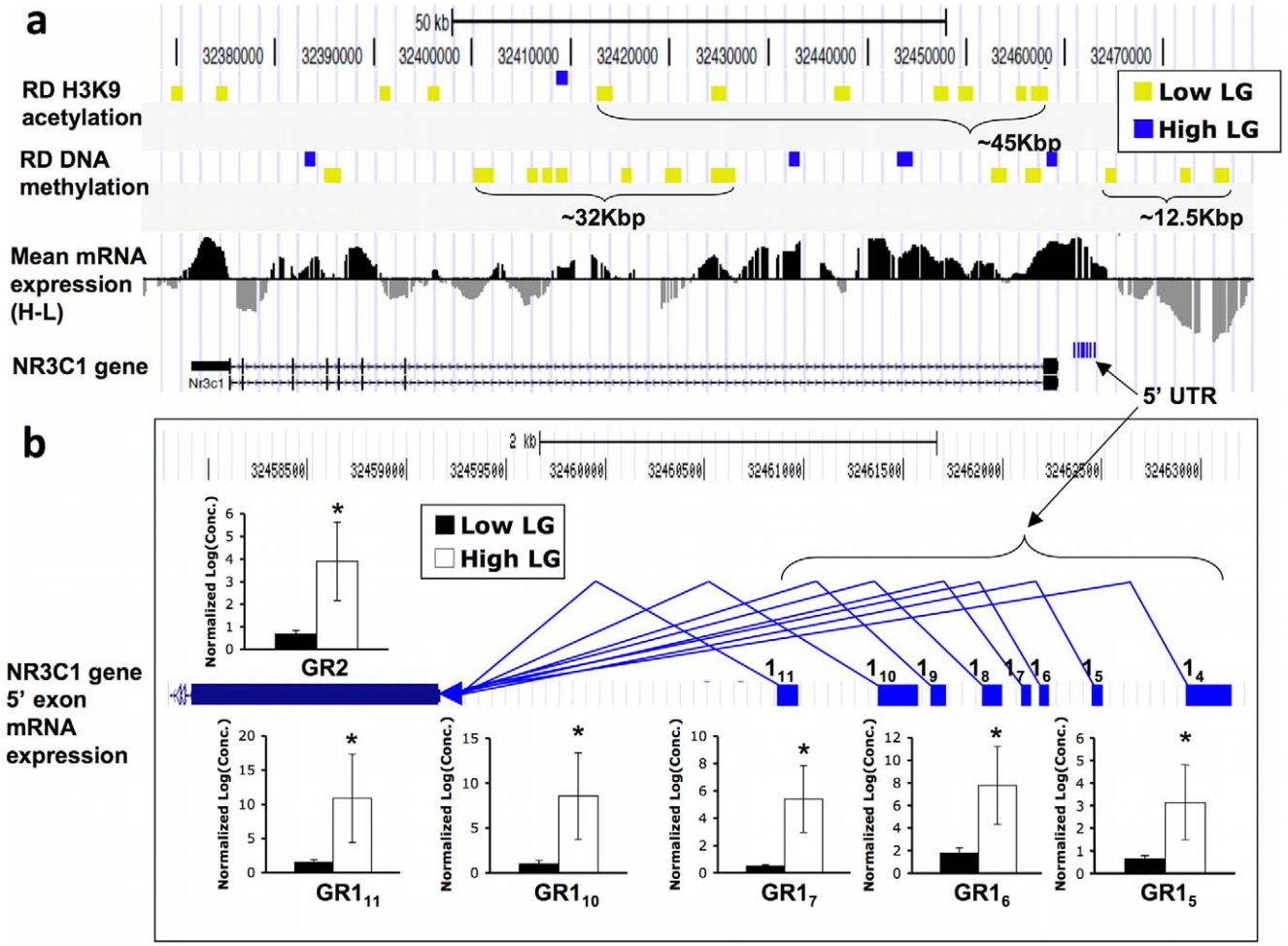

Figure 4. Epigenomic neighborhood of the glucocorticoid receptor gene. (**a**) Track of the glucocorticoid receptor gene, *NR3C1*, showing examples of large RDme/ac throughout the 5′ end and intron of the gene. Individual tracks, from the top to the bottom, show locations of hyperacetylated (blue) and hypoacetylated (yellow) RDac in High relative to Low LG adult offspring, RDme displayed in the same manner, mean gene expression, and the location of the *NR3C1* gene. (**b**) Schematic representation splice variant assembly of 5′ untranslated elements as well as the first coding exon (GR2) of the *NR3C1* gene, along with gene expression differences between High and Low LG adult offspring (* = $P<0.05$). Each real-time PCR reaction was performed in triplicate and results are displayed as mean +/− SEM.
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that shows a cluster-wide response in DNA methylation and expression and exhibits the highest number of RDme relative to other regions in the locus: the -α, -β, and -γ protocadherin (Pcdh) gene clusters (82 of 696 RDme; $P = 0.006$, permutation test). Among Low LG offspring, we observed a significant enrichment for hypermethylated RDme across the entire Pcdh gene cluster (45 of a total of 350 RDme hypermethylated in Low LG offspring were found within the Pcdh gene clusters; $P = 0.01$, permutation test).

Pcdh genes are predominantly expressed in neurons at synaptic junctions, and the assembly of these cell surface proteins is regulated by differential promoter activation and alternative pre-mRNA splicing [31]. Although the mechanisms underlying differential promoter activation are not well understood, promoter DNA methylation and histone acetylation play a role in Pcdh gene silencing [32,33]. Consistent with this hypothesis, Pcdh gene expression induced in response to High LG maternal care is accompanied by higher in exonic H3K9 acetylation and DNA methylation ($P<1\times10^{-300}$ for both by Wilcoxon rank sum test) and lower proximal promoter DNA methylation in a majority (17 of 23, or 74%) of Pcdh genes showing a significant increase in expression among High LG compared to Low LG offspring (**Fig. 5**). High LG offspring show a significant increase in transcription in 20 Pcdh of 33 genes profiled within the Pcdh gene clusters (**Table S1**). Taken together, these results showing a transcriptional and epigenetic response to maternal care across the Pcdh gene family suggest that the epigenomic response to maternal care may act coordinately on a family of genes localized in the same broad genomic region.

Discussion

The quality of maternal care in rodents has a widespread impact on phenotype that persists into adulthood, providing a model to study epigenetic mechanisms mediating the impact of the early life social environment on health later in life [6]. In this study, we asked whether our traditional approach examining the regulatory elements of candidate genes reflects the totality of the epigenetic response to naturally occurring environmental stimuli. By extending our analysis beyond the predicted boundaries of the *NR3C1* gene using high-density coverage of megabases of sequence, we investigated whether changes are limited to a small

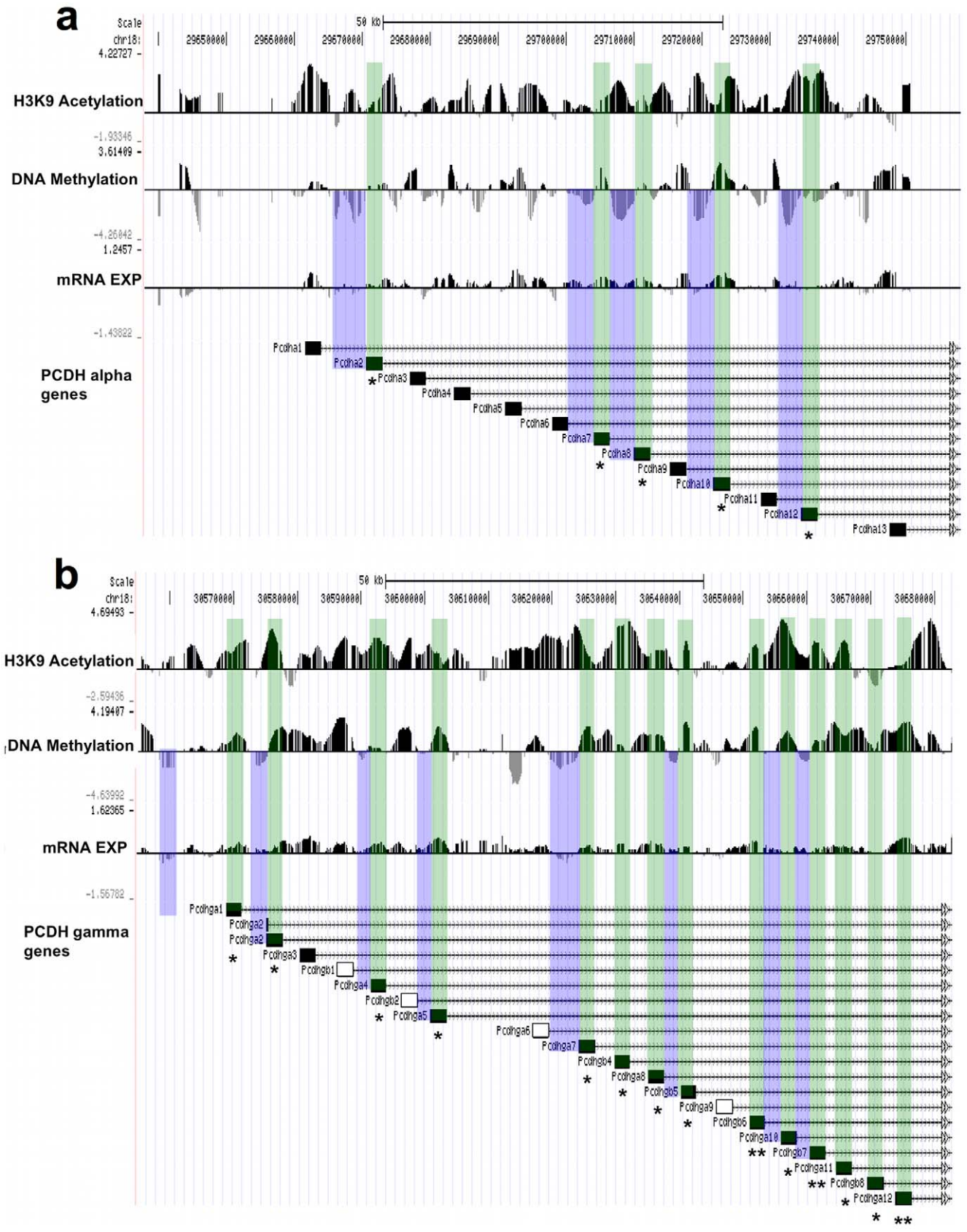

Figure 5. Epigenomic neighborhoods of the first exons in protocadherin gene clusters. Genes with hypomethylated 5′ gene ends (blue), hypermethylated and hyperacetylated exons (green) and significantly greater gene expression among High compared to Low LG adult offspring (H–L) are shown for (**a**) protocadherin-α, (**b**) protocadherin-γ gene clusters. Gene expression differences of genes surveyed by quantitative RT-PCR (filled boxes; ** = $P<0.01$, * = $P<0.05$) are shown relative to the location of other nearby Pcdh genes within each cluster (unfilled boxes).
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number of candidate genes, whether the changes are limited to 5′ regulatory regions and whether they are exclusive to regions encoding mRNAs.

We found non-random patterns of epigenetic and transcriptional alterations in a number of genes in association with differences in maternal care (**Fig. 2**). The specificity of this pattern is further underscored by the fact that both increased and decreased peaks of acetylation and DNA methylation are observed throughout the region. However, the response is gene-specific, as not all genes appear to respond to differences in maternal care (**Table S1**). Differences in broad epigenetic marks co-cluster over large distances (**Fig. 3a**), supporting previous work in cancer cells and suggesting the possibility of widespread epigenetic effects on multiple genes in the same genomic regions in response to maternal care. Analysis of our data with respect to protein-coding genes reveals expected relationships between epigenetic marks and gene expression levels. Increased transcription is associated with decreased 5′ DNA methylation and increased exonic H3K9 acetylation and DNA methylation (**Fig. 3b–c**). Previous studies have examined the relationships between differences in DNA methylation and histone acetylation and gene expression in on/off states of gene expression activity, as seen in cancer and cellular differentiation paradigms[23,25,26,27,29]. Our data suggest that the *modulation* of gene expression in response to environmental stimuli follows the same rules.

Our approach using high-density tiling microarrays also provided us with a "macroscopic" perspective of the epigenetic and transcriptional responses to maternal care. By zooming out of the specific suspected regions, we discovered differentially-methylated and acetylated regions that span large domains of sequence in the vicinity of the *NR3C1* gene (**Fig. 4a**). Among adult offspring of animals that had received relatively low levels of maternal care, we identified several hypermethylated RDme and hyperacetylated RDac upstream of the 5′ NR3C1 exon variants as well as in intronic regions, where transcription was also detected. These results suggest the possible involvement of non-coding RNAs and alternative splice variants in response to maternal care. Future studies are required to determine whether these broad regions that are differentially methylated in response to maternal care regulate NR3C1 expression.

Although when examined individually, different regions exhibit highly specific responses (**Fig. 4b**; **Fig. 5a–b**), large-scale patterns emerge when we use a "macro" view of the entire chromosomal region (**Fig. 2**). Both increased and decreased peaks of acetylation and DNA methylation are observed throughout the region. In addition, of the 29 transcripts showing statistically significant differences in transcription, all are significantly more expressed among High LG adult offspring (**Table S1**). These data indicate specificity in transcriptional changes at the single gene level as well as an overall common response at a "large-scale" level consisting of many neighboring genes. The fact that the observed response is a result of a naturally-occurring variation in maternal care rather than average "static" levels of histone acetylation and DNA methylation points to the possibility that a long-range coordinated regulation of genome function may play a role in the long-term programming of the genome.

One possible role for clustering of epigenetic responses across wide areas is the coordinate regulation of a large group of functionally related genes. We discovered that the expression of a large cluster of the Pcdh genes is coordinately regulated with respect to maternal care. Remarkably, the increase in gene expression in the High maternal care group spans genes within each of the Pcdh gene clusters (**Fig. 1c**; **Fig. 5a–b**). This family of genes correspondingly contains a significant overrepresentation of differentially methylated regions. It is interesting to speculate that the Pcdh gene family may have evolved though gene duplication as a class of functionally-related genes under coordinate epigenetic regulation. Indeed, coordinated silencing of the Pcdh family of genes was seen in cancer [34]. However, in cancer, processes related to cell-selection might be involved in a progressive spreading of DNA methylation [35,36,37].

We recognize that we do not yet know whether differences in Pcdh gene expression play a role in the effects of maternal care on brain function in offspring. Future studies are needed to examine the consequences of the epigenetic regulation of Pcdh gene expression for the regulation of Pcdh protein and downstream functional effects. Pcdh genes are preferentially expressed in neurons, including the hippocampus, and regulate synaptic development and function [38]. Pcdh-α gene expression during rodent neural development is highest in early postnatal life (until PD21), when it is involved in specifying the innervation of serotonergic neurons in the hippocampus [39]. Studies indicate enduring influences of differences in maternal care on hippocampal neuroplasticity, including effects on LTP [40,41] and synaptic morphology [42,43]. Indeed, a rich literature suggests widespread effects of the prenatal and postnatal environment on the developing brain (for reviews see [44,45]). For example, whereas other maternal factors such as maternal stress during pregnancy induce long-term influences on behavior, including hippocampally-mediated fear conditioning and spatial learning, adoption studies show that postnatal maternal care can reverse these effects [11,13,46]. Both hippocampal synaptic density and LTP as well as contextual fear conditioning and spatial learning vary as a function of maternal care in the rat [10,15]. It is interesting to speculate that differences in Pcdh gene regulation may be functionally relevant for hippocampal development.

The mechanisms responsible for this coordinated epigenomic response and its maintenance into adulthood are unknown. We observed a broad epigenomic response associated with an extensive difference in gene expression. These broad epigenomic and transcriptome changes occurred not in response to disease (e.g. cancer) [34] or artificial interventions (e.g., gene knock-out or exposure to toxins), but in the context of a natural variation in maternal behavior. Although the changes are broad, not all genes are affected. The specificity of the response and its pattern are consistent with the hypothesis that the epigenetic response is indeed a biological signal. Our data suggest that epigenetic variations in the context of early life environment variations and perhaps other environmental influences involve coordinate changes in gene-networks rather than dramatic changes in a single or few genes. Our data also suggest that this response may involve more than protein coding mRNAs. Our traditional approaches to examine relationships between epigenetic regulation, gene function and phenotype were developed to examine changes within genetic elements defined apriori (promoters, exons, 3′ gene ends) in single or few candidate genes. If, in addition, the response to an environmental stimulus such as maternal care involves more widespread or coordinated changes across multiple genomic regions, new experimental approaches are needed to examine the contribution of these changes to the ultimate phenotype. Our data suggest multiple levels of variations in DNA methylation and H3K9 acetylation, from site-specific gene-specific responses as previously reported [6,17] to the regional responses shown in this study. Although future experiments are needed to address the relative role of "micro" and "macro" epigenetic responses, these data suggest that the broad epigenetic regulation of gene expression may form part of a coordinated response to early maternal care.

Materials and Methods

Ethics Statement

All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and the protocol was approved by the McGill University Animal Care Committee, permit number 3284.

Subjects and tissue preparation

Three to 4 animals per group were used in all microarray and quantitative immunoprecipitation experiments. An additional cohort of 8 animals per group was used for gene expression analysis. A maximum of 2 animals from any one litter were used, to control for possible effects attributable to variation between litters rather than variation as a function of High and Low LG. The animals were Long-Evans hooded rats born in our colony originally obtained from Charles River Canada (St. Constant, Québec). Maternal behavior was scored by using a version of the procedure described elsewhere[16]. Hippocampal tissue was dissected from 90-day-old (adult) male High and Low LG offspring and stored at -80°C. Genomic DNA extraction (DNeasy, Qiagen) and quantification (Nanodrop ND-1000 spectrophotometer, Thermo Scientific) as well as RNA extraction (RNeasy plus, Qiagen) and quality assessment (Bioanalyzer 2100, Agilent) were performed according to the manufacturer's protocol (see **Methods S1** for details).

Chromatin/DNA immunoprecipitation and microarray hybridization

The procedure for methylated DNA immunoprecipitation was adapted from previously published work,[47,48,49] and H3K9 acetylation ChIP assays[50] were performed using the ChIP assay kit protocol (06-599, Upstate Biotechnology), as previously described[6]. The amplification (Whole Genome Amplification kit, Sigma) and labeling reaction (CGH labeling kit, Invitrogen), and all the steps of hybridization, washing and scanning were performed following the Agilent protocol for chip-on-chip analysis (see **Methods S1** for details). Three animals per group were used in the immunoprecipitation microarray experiments, and microarrays were hybridized in triplicate for each sample.

cDNA microarray hybridization

RNA spike-in controls (Agilent) were added to RNA prior to generating cDNA. The cDNA was amplified and labeled with Cy3 or Cy5 (GE Healthcare) according to manufacturer's instructions (Fairplay III, Agilent; See **Methods S1** for details). Four animals per group were used for the gene expression microarrays, and a dye-swap experiment was performed for each subject in duplicate.

Microarray design and analysis

Custom 44 K tiling arrays were designed using eArray (Agilent technologies). Probes of approximately 55 bp were selected to tile all unique regions within approximately 3.5 MB upstream and downstream of the *NR3C1* gene described in Ensembl (version 44) at 100 bp-spacing. Probe intensities were extracted from hybridization images using Agilent's Feature Extraction 9.5.3 Image Analysis Software and analyzed using the R software environment for statistical computing[51]. Log-ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray. Each microarray was normalized using quantile-normalization[52] assuming an identical overall distribution of measurement across all samples. Gene expression levels were estimated as the mean probe values across exons. DNA methylation and H3K9 acetylation levels at genomic locations were estimated using a Bayesian convolution algorithm to incorporate probe values from nearby probes[53]. Gene expression differences associated with maternal care were obtained using RMA[54] applied to sample probe values inside exons. RDme/ac were identified by computing a modified t-statistic for each probe and then significant levels of agreement across 1000 bp regions. Enrichment of RDac and RDme was determined by comparing base-pair overlap of these regions with overlap of randomly selected RDac/RDme (see **Methods S1** for details). All microarray data are MIAME compliant and the raw data have been deposited in Gene Expression Omnibus (GEO) at NCBI (www.ncbi.nlm.nih.gov/geo/), accession number pending.

Quantitative real-time PCR of immunoprecipitated samples (qCHIP)

Gene-specific real-time PCR validation of microarray was performed in an identical manner for H3K9 acetylation and DNA methylation enrichment[21] for the same subjects used for microarray experiments (n = 3/group; see **Methods S1** for details). Relative enrichment of triplicate reactions were determined as a ratio of the crossing point threshold (Ct) of the amplified immunoprecipitated fraction (with either anti-histone H3K9 acetylation or anti 5-meC antibody) over the Ct of the amplified input genomic DNA fraction according to the formula: IP(Ct)/IN(Ct). The calculated immunoprecipitation enrichment was plotted and standard error bars were displayed.

Sodium bisulfite mapping of DNA methylation

Sodium bisulfite mapping was performed as previously described[55]. After gene-specific PCR amplification (**Table S2**) of sodium bisulfite treated DNA for each subject, a mix of 10 ng of the gel-extracted PCR product from all of the subjects from each High and Low LG group (n = 3/group) were used for subsequent molecular cloning (Cequation 8800, Beckman-Coulter). We obtained 20 clones for sequencing from 2–3 independent PCR reactions for each subject.

Genotyping

The genes verified for differences in DNA methylation by qCHIP analysis were further analyzed for genotyping using identical primers (**Table S2**). The resulting PCR products for each subject were sequenced bidirectionally using the forward and the reverse primer by Genome Quebec (ABI 3100, Applied Biosystems). Genetic variation was assessed throughout the PCR amplicon used for qCHIP analysis by alignment of genomic DNA with the published gene sequence (CLC Workbench, CLC bio).

Quantitative real-time RT-PCR analysis

The expression patterns of 45 transcripts examined by microarray were quantified. For genes shown in **Figure 1c** (also see **Table S1**) primer design (**Table S2**) and analysis were performed by Genome Quebec (ABI lightcycler, ABI biosystems), whereby the expression of an additional 7 housekeeping genes (Actb, Gapdh, Gusb, Pum1, Rpl19, Rps18, Tubb5) was assessed for the same subjects used for microarray hybridization. The gene showing the least variance between High and Low LG adult offspring was selected as the reference gene for all subjects (GusB), and statistical significance, fold differences and standard errors of the mean were calculated according to published methods using the freely-available Relative Expression Software Tool program[56]. For the quantification of gene expression differences related to the *NR3C1* gene shown in **Figure 4b** and the Pcdh gene

clusters shown in **Figure 5** (also see **Table S1**), a standard curve was generated from 7 serial dilutions of a mixture of cDNA from each High and Low LG offspring, and gene expression was quantified relative to the tubulin housekeeping gene (480 lightcycler, Roche) for an additional cohort of 8 High LG and 8 Low LG offspring, according to previously published methods (**Table S2**; [19,57]). All reactions for all genes were performed in triplicate and statistical significance was determined as $P<0.05$ using one-tailed t-tests.

Supporting Information

Methods S1
Found at: doi:10.1371/journal.pone.0014739.s001 (0.08 MB PDF)

Table S1 Genes with higher expression in High LG offspring. Listed are the fold change for 44 transcripts selected for gene expression profiling (High LG/Low LG). Expression is significantly higher in the High LG offspring for 29 transcripts (** = $P<0.01$, * = $P<0.05$). Also shown are distances to the nearest RDme and RDac both before and after the transcription start site of each gene, and whether they are hyper-methylated/acetylated or hypo-methylated/acetylated in High relative to Low LG adult offspring.
Found at: doi:10.1371/journal.pone.0014739.s002 (0.04 MB XLS)

Table S2 Sequence information for primers used for H3K9 acetylation, DNA methylation, and gene expression validation of microarrays.
Found at: doi:10.1371/journal.pone.0014739.s003 (0.04 MB XLS)

Figure S1 Pearson correlation between DNA methylation levels estimated from microarray data and levels estimated from qChIP for each gene validated by quantitative real-time PCR (red circles).
Found at: doi:10.1371/journal.pone.0014739.s004 (0.07 MB TIF)

Figure S2 DNA methylation validated by sodium bisulfite mapping showing expected enrichment of DNA methylation in Low (black bars) compared to High LG (white bars) animals for the majority of CpG sites examined. These data confirm the enrichment in Low LG relative to High LG offspring estimated from microarray and qChIP.
Found at: doi:10.1371/journal.pone.0014739.s005 (0.10 MB TIF)

Figure S3 DNA methylation, H3K9 acetylation and gene expression levels. (a) Average levels of H3K9 acetylation and DNA methylation across all regions, and gene expression levels within protein coding exons only for all subjects are depicted across the 7Mb region centered at the NR3C1 gene (see Supporting Methods for calculation of levels). (b) Levels across gene-associated regions for all genes are depicted. (c) Levels are depicted across CpG islands (H3K9 acetylation levels are red, DNA methylation levels are blue, and gene expression levels are green). All data are mean values and line thickness denotes SEM.
Found at: doi:10.1371/journal.pone.0014739.s006 (0.30 MB TIF)

Figure S4 An example of predicted nucleosome occupancy and actual H3K9 acetylation levels estimated from microarray data for Protocadherin-α genes. Predictions were obtained in silico solely from DNA sequence using a previously published tool [1].
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Author Contributions

Conceived and designed the experiments: POM MS AS MH MJM MS. Performed the experiments: POM AS TCTH. Analyzed the data: POM MS AS. Wrote the paper: POM MS AS MH MJM MS.

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Research Report

Impaired social recognition memory in recombination activating gene 1-deficient mice

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ABSTRACT

The recombination activating genes (RAGs) encode two enzymes that play key roles in the adaptive immune system. RAG1 and RAG2 mediate VDJ recombination, a process necessary for the maturation of B- and T-cells. Interestingly, RAG1 is also expressed in the brain, particularly in areas of high neural density such as the hippocampus, although its function is unknown. We tested evidence that RAG1 plays a role in brain function using a social recognition memory task, an assessment of the acquisition and retention of conspecific identity. In a first experiment, we found that RAG1-deficient mice show impaired social recognition memory compared to mice wildtype for the RAG1 allele. In a second experiment, by breeding to homogenize background genotype, we found that RAG1-deficient mice show impaired social recognition memory relative to heterozygous or RAG2-deficient littermates. Because RAG1 and RAG2 null mice are both immunodeficient, the results suggest that the memory impairment is not an indirect effect of immunological dysfunction. RAG1-deficient mice show normal habituation to non-socially derived odors and habituation to an open-field, indicating that the observed effect is not likely a result of a general deficit in habituation to novelty. These data trace the origin of the impairment in social recognition memory in RAG1-deficient mice to the RAG1 gene locus and implicate RAG1 in memory formation.

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1. Introduction

The RAG genes have been the subject of intense study in the immune system, where they mediate the diversification of B- and T-cell receptors via somatic recombination. During somatic recombination, genetic "recombination signal sequences" are targeted by RAG1 and RAG2 enzymes, which together cleave variable, diversity, and joining (VDJ) gene segments located on several chromosomes. These are brought together during a process of DNA rearrangement by DNA repair mechanisms. The DNA rearrangement initiated by RAG occurs in a combinatorial fashion, greatly increasing the variety of B- and T-cell receptor subtypes. As a result of this and other processes, the immune system is able to recognize

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virtually any foreign pathogen. A number of researchers have speculated that similar processes of receptor diversification may occur in the central nervous system (CNS) (Pena De Ortiz and Arshavsky, 2001; Schatz and Chun, 1992; Yagi, 2003), in part because many of the same molecules involved in somatic recombination, such as those involved in DNA double-strand break repair, are necessary for neural development and function (Chun and Schatz, 1999a; Chun and Schatz, 1999b; Colon-Cesario et al., 2006). However, direct evidence of somatic recombination in the CNS remains to be demonstrated.

Intriguingly, RAG1 but not RAG2 is expressed in the CNS, the only tissue outside of the immune system shown to express RAG (Chun et al., 1991). RAG1 is expressed in both embryonic and postnatal brain, with expression highest in limbic areas, including in the hippocampus, and in the cerebellum (Chun et al., 1991; Sun et al., 2007). These are areas of high neural density, suggesting RAG1 may be present in neurons. The immunological consequence of RAG1 or RAG2 deficiency is an obvious severe combined immunodeficiency (Mombaerts et al., 1992; Shinkai et al., 1992). However, RAG1-deficient mice show no obvious alterations in CNS anatomy or physiology (Chun et al., 1991). The only other previous study to directly address functional alterations as a result of RAG1-deletion (Cushman et al., 2003) reported increased activity but there was no effect of RAG1-deletion on water maze memory, pre-pulse inhibition, or acoustic startle response, standard measures of neurobehavioral function assessing limbic circuitry.

In the present study, we examined the performance of mice with RAG1-deletion (RAG1KO) on a social recognition memory task. The social recognition memory task assesses the ability of rodents to successfully identify previously encountered conspecifics, mainly via olfactory cues (Gheusi et al., 1994). In socially-housed mice, social recognition memory at delay intervals longer than 30 min following exposure to a conspecific depends upon intact hippocampal function (Kogan et al., 2000). A variety of social recognition memory paradigms have been developed with differing memory performance depending on the particular task parameters (Markham and Juraska, 2007). We sought to take advantage of the social recognition memory task's rapid implementation, as an example of a so-called "one trial learning" task and one that is amenable to pathogen-free testing conditions for use with immunocompromised subjects.

2. Results

2.1. *Experiment 1: social recognition memory among RAG1KO and RAG1 wildtype (WT) mice*

A social recognition memory task, consisting of an initial exposure to an ovariectomized "stimulus mouse" and a subsequent discrimination trial where subjects were simultaneously exposed to the same stimulus mouse and a novel stimulus mouse, was employed to examine memory among RAG1KO and WT mice. Preliminary experiments during the dark phase of the cycle in our facility using WT mice indicated that social recognition memory was intact after a 30 min delay but was not evident after 120 min delay. We therefore assessed discrimination of the previously exposed stimulus mouse after delays of 30 min, 60 min, and 120 min. Separate cohorts of mice were used at the 30 min ($n=12$ R1KO and $n=12$ WT), 60 min ($n=16$ R1KO and $n=16$ WT) and 120 min trials ($n=12$ R1KO and $n=12$ WT). Subjects that repeatedly fought with or attempted to mount the non-receptive stimulus mouse were removed from analysis (30 min: $n=2$ R1KO and $n=2$ WT; 60 min: $n=2$ R1KO and $n=1$ WT; 120 min: $n=1$ R1KO and $n=1$ WT).

There were no statistically significant differences in investigation of the stimulus mouse during the initial encounter between RAG1KO and WT mice on either the 30 min, 60 min, or 120 min trials (Fig. 1A; $P>0.05$), suggesting that differences in duration of exposure to the stimulus mouse were unlikely to influence subsequent recognition memory performance. After a delay of 30 min, both genotypes successfully recognized the previously exposed stimulus mouse (Fig. 1B, left panel; RAG1KO: $t(9)=2.39$, $P=0.040$, $d=1.11$; WT: $t(9)=3.28$, $P=0.009$, $d=1.47$). At the 60 min delay, RAG1KO mice showed impaired social recognition memory (Fig. 1B, middle panel; $P>0.05$) whereas memory among WT mice was intact ($t(14)=4.93$, $P=0.0001$, $d=1.87$). After a delay of 120 min, both genotypes did not show memory for the stimulus mouse (Fig. 1B, right panel; $P>0.05$).

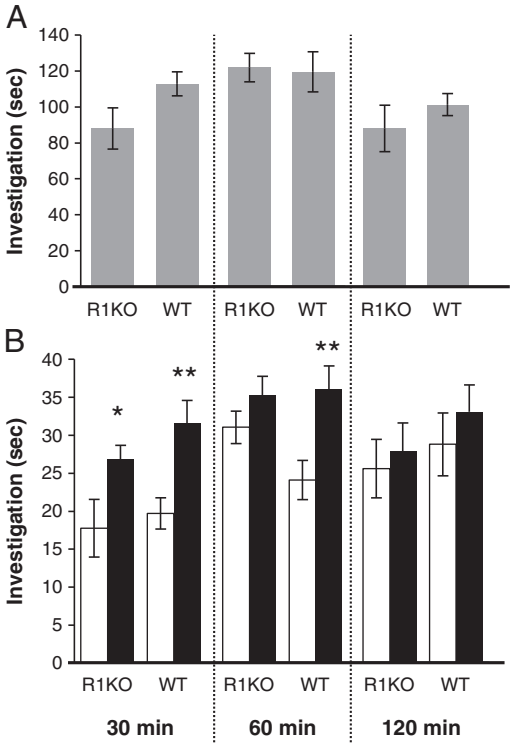

Fig. 1 – Experiment 1: social recognition memory in RAG1KO and RAG1WT mice. (A) Duration of investigation of the stimulus mouse on the initial encounter. (B) Duration of investigation on the discrimination trial towards the same previously encountered (white bars) and a novel (black bars) stimulus mouse after delay intervals of 30 min, 60 min, and 120 min. Data are mean ± SEM; *$P<0.05$, **$P<0.01$.

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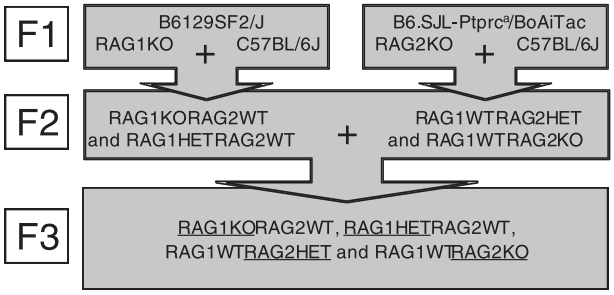

Fig. 2 – Overview of the breeding strategy used to generate F3 "intercross" mice for the behavioral experiments. Underlines denote nomenclatures of the adult male littermate genotypes used for experiments described in Figs. 3–5.

2.2. *Experiment 2: social recognition memory among "intercross" mice*

It is not clear from experiment 1 whether the behavioral differences observed between RAG1KO and WT mice reflected impaired learning and memory per se or the effects of immunodeficiency (i.e., sickness). We therefore undertook extensive breeding that homogenized potential differences in background genetics, maternal factors, and secondary effects of immunodeficiency by generating "intercross" mice from an F1 stock of RAG1- and RAG2-deficient animals (Fig. 2; also see section 4.1 of Experimental procedures). RAG1KO, RAG1HET, RAG2KO, and RAG2HET littermates of homogenized background genetics were used in subsequent experiments. Whereas RAG2KO mice show exactly the same immunodeficient phenotype as RAG1KO, only RAG1 was shown to be expressed in the brain (Chun et al., 1991; Sun et al., 2007). Also, as heterozygosity for either RAG gene deletion is known to confer normal immunological function, heterozygous animals served as a control for immunodeficiency. The use of two heterozygous genotypes (RAG1HET and RAG2HET) allowed for the examination of possible gene dosage effects among RAG1-deficient mice (i.e., a greater memory impairment among RAG1HET compared to RAG2HET could indicate effects of RAG1 hyposufficiency in the brain).

A single cohort of $n=12$ animals of each genotype were used to assess social recognition memory performance at all delay intervals, where $n=1$ RAG1HET, $n=2$ RAG1KO, $n=4$ RAG2HET, and $n=1$ RAG2KO mice were excluded from analysis due to repeated fighting with or attempting to mount the non-receptive stimulus female. As in experiment 1 above, there were no significant differences between genotypes in the duration of investigation during the initial encounter with the stimulus mouse on either the 30 min, 60 min, or 120 min trials (Fig 3A; $P>0.05$). After a delay of 30 min, all genotypes successfully recognized the previously exposed stimulus mouse (Fig. 3B, left panel; RAG1HET: $t(10)=3.19$, $P=0.004$, $d=1.42$; RAG1KO: $t(9)=2.69$, $P=0.025$, $d=1.23$; RAG2HET: $t(7)=3.74$, $P=0.007$, $d=1.93$; RAG2KO: $t(10)=2.32$, $P=0.043$, $d=1.03$). After a delay of 60 min, RAG1KO mice showed impaired memory for the stimulus mouse (Fig. 3B, middle panel; $P>0.05$), whereas memory was intact among the other genotypes (RAG1HET: $t(10)=1.81$, $P=0.041$, $d=1.00$; RAG2HET: $t(7)=1.89$, $P=0.009$, $d=1.79$; RAG2KO: $t(10)=2.56$, $P=0.028$, $d=1.10$). After a delay of 120 min, none of the genotypes showed significant memory for the stimulus mouse (Fig. 3B, right panel; $P>0.05$).

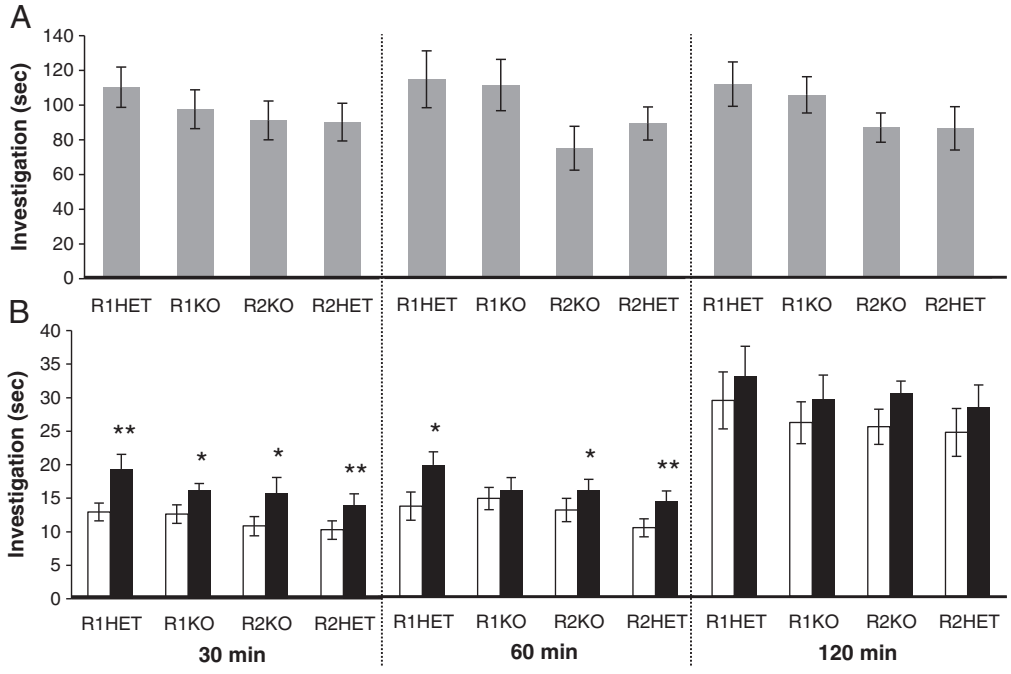

Fig. 3 – Experiment 2: social recognition memory in RAG1RAG2 intercross littermates. (A) Duration of investigation of the stimulus mouse on the initial encounter. (B) Duration of investigation on the discrimination trial towards the same previously encountered (white bars) and a novel (black bars) stimulus mouse after delay intervals of 30 min, 60 min, and 120 min. Data are mean ± SEM; $^{*}P<0.05$, $^{**}P<0.01$.

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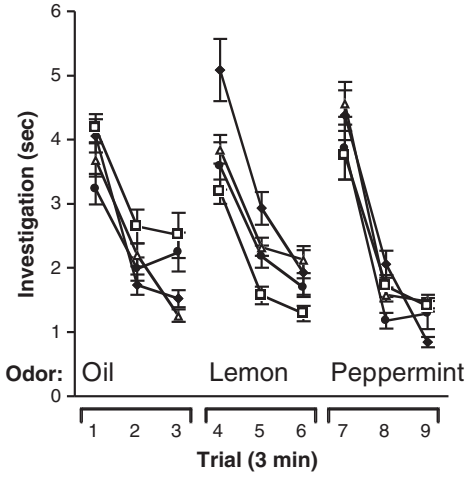

Fig. 4 – Olfactory habituation. The duration of investigation is shown for RAG1KO (filled circles), RAG1HET (open triangles), RAG2KO (filled diamonds), and RAG2HET (open squares) intercross littermates when presented with sesame oil, 1% lemon scent in sesame oil, and 1% peppermint scent in sesame oil over 9 consecutive trials of 3 min duration.

2.3. *Olfactory habituation*

To assess whether RAG1KO mice show impaired olfactory function in the absence of socially-derived odor cues, intercross subjects were tested in an olfactory habituation task (Guan et al., 1993; Schellinck et al., 1992; Schellinck et al., 2001). The cohorts of mice from experiment 2 above were used. All mice showed the ability to discriminate non-socially-derived odors (Fig. 4). There were no significant differences in investigation between genotypes nor was there a significant interaction between genotype and trial ($P>0.05$). However, there were significant differences in investigation duration among each of the 9 trials ($F_{(2,\ 91)}=16.49$, $P<0.0001$). Post-hoc testing revealed that investigation on the first trial of each novel odor presentation (i.e., trials 1, 4, and 7) was significantly greater than investigation on the second and third trials for each odor, suggesting that all mice showed habituation to the continued presentation of the same odor (all, $P<0.05$). Investigation on trials 4 and 7 was also significantly higher than on the preceding trial ($P<0.05$), indicating that all mice showed dishabituation to the presentation of each novel odor.

2.4. *Open-field behavior*

A previous report suggested that RAG1KO mice show impaired habituation to an open-field in comparison to WT controls (Cushman et al., 2003). To assess differences in habituation to a novel environment among RAG1KO mice and littermate controls, a naïve cohort of $n=19$ RAG1HET and $n=17$ RAG1KO "intercross" adult male mice were used. In contrast to previously reported findings (Cushman et al., 2003), RAG1KO and RAG1HET mice showed similar rates of habituation in the open-field (Fig. 5). There was no significant main effect of genotype or interaction between time and genotype ($P>0.05$); however, the effect of time was highly significant ($F_{(11,\ 374)}=32.15$, $P<0.0001$), suggesting that, like RAG1HET mice, RAG1KO mice show significant habituation in locomotor activity over time when exposed to a novel open-field. Similarly, while there was no significant main effect of genotype or interaction between time and genotype ($P>0.05$), there was a highly significant main effect of time in the number of entries into the center of the open-field ($F_{(11,\ 374)}=28.51$, $P<0.0001$). Post-hoc testing revealed no significant differences between genotypes at any of the time intervals examined ($P>0.05$). As the number of center entries in a novel open-field is commonly used as a measure of anxiety-like behavior (Post et al., 2010), these data suggest that RAG1KO and RAG1HET exhibit similar levels of anxiety-like behavior in the open-field task.

3. Discussion

This study reports evidence that social recognition memory is impaired in RAG1KO mice. RAG1KO mice showed the ability to

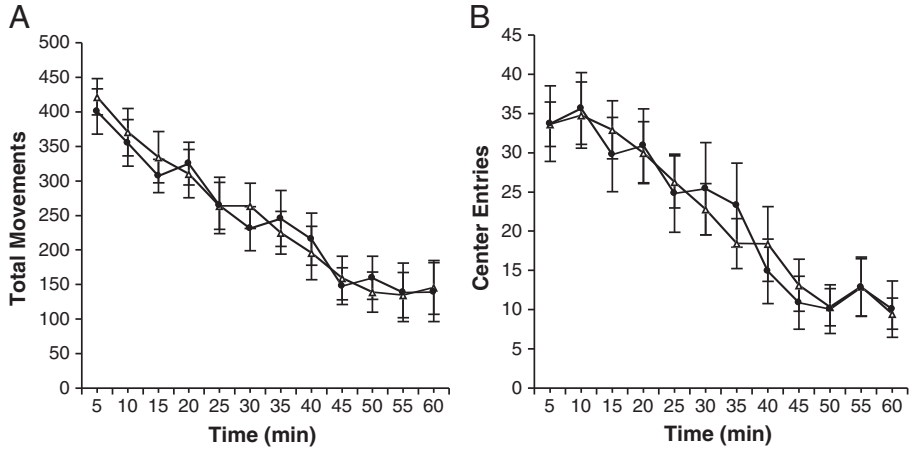

Fig. 5 – Open-field behavior. Locomotor activity among RAG1KO (filled circles) and RAG1HET mice (open triangles) intercross littermates. (A) The total number of movements is shown over each consecutive 5 min time block for the 60 min trial duration. (B) The total number of entries to the center of the open-field is shown over each consecutive 5 min time block for the 60 min trial duration.

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remember a previously exposed conspecific after a delay of 30 min but were impaired relative to controls at a 60 min delay interval. This finding was replicated in separate cohorts of animals, reflecting the robustness of the effect (Figs. 1 and 3). Although not germane to the question of whether social recognition memory had occurred, absolute levels of investigation on the discrimination trials appeared somewhat lower in the 30 min and 60 min delay intervals compared to the 120 min intervals in experiment 2. Given the length of the 120 min delay interval, it is possible that this difference reflected dishabituation to the testing procedure at the longest delay under conditions used in experiment 2, whereas higher levels of investigation at 30 min and 60 min delay intervals in experiment 1 reflected a ceiling effect of high levels of investigation.

The impairment in social recognition memory in RAG1KO mice relative to RAG1HET and RAG1WT mice was observed even among littermates, where background genotype and maternal factors were controlled (Fig. 3). An important finding in our study was that of intact social recognition memory in RAG2KO littermates, providing evidence that the observed effects do not likely result from secondary effects of immunodeficiency. We did not find evidence of a gene dosage effect, as RAG1HET mice showed similar social recognition memory performance as RAG2HET mice. These data support the view that RAG1-deficiency in the brain leads to impaired memory.

It is plausible that exploratory behavior by the familiar stimulus mouse on the discrimination trial may influence social recognition memory performance by virtue of the *stimulus mouse*'s memory for the subject. As an explanation for our findings, we cannot rule out the possibility that the subject's olfactory signature was in some way altered as a result of RAG1-deletion, affecting the behavior of the stimulus mouse. However, we feel that this possibility is unlikely for at least three reasons. First, as virgin ovariectomized females, the stimulus mice were not sexually responsive and showed little investigative behavior of the male subjects, whereas each male subject showed robust investigation of the stimulus mice. Indeed, instances where either the novel or the familiar stimulus mouse engaged in active investigation of the subject were exceedingly rare in this study, precluding quantitative analysis, and such instances were not included in measures of the subject's investigatory behavior. Second, whereas subjects were habituated to a novel cage for at least 20 min prior to the start of testing, reducing the subject's locomotor responses to the novel home-cage environment, stimulus mice were introduced into the subjects' cage at the start of each trial. In this respect, the task was similar to the resident–intruder task, where aggressive behavior of the subject towards a "stimulus mouse" introduced into the subject's home-cage is measured (Bartolomucci et al., 2009), although the use of group-housed animals in this task precluded the testing in the subject's home-cage. Third, on a given testing day, the stimulus mice were alternately presented to the subjects during the "initial encounter" trials. As a result, at a retention interval of 60 min where differences between genotypes were detected, a given stimulus mouse had been presented to approximately 5–6 subjects during successive initial encounters (i.e., a new subject every 8–10 min throughout the delay interval) prior to re-encountering the same subject and the novel stimulus mouse on the discrimination trial. Thus, in order for the stimulus mouse to show a preference for the previously exposed subject, the stimulus mouse would have to maintain memory for the same subject during an increasing number of trials. Future studies are needed to examine the effects of such retroactive interference in this task.

In comparison to another recently published study (Noack et al., 2010) where social recognition memory was reported 24 h after an initial encounter, in our procedure, social recognition memory was not evident after a 2 h delay. As noted, preliminary studies in our facility yielded comparable results in additional wildtype mice. Although some reports indicate that mice are able to form long-lasting social recognition memory after a single exposure to a stimulus mouse (Kogan et al., 2000), our procedure differed in several ways. First, we conducted our studies in the dark "active" phase of the circadian cycle. It should be noted that absolute duration of investigation on the initial encounter in our study is on the order of 2-fold higher than that reported by Noack et al. (2010) It is possible that such high levels of investigation, observed in all of the genotypes examined, obscured "memory" for the previously exposed conspecific as a result of increased levels of active investigation rather than a deficit in memory per se. Also, in contrast to Noack et al. (2010), we used ovariectomized adult females as stimulus mice instead of juvenile conspecifics. Although ovariectomized females are routinely used in social recognition memory experiments (Ferguson et al., 2000; Gheusi et al., 1994), it is possible that these stimulus animals do not elicit the same levels of habituation with repeated exposure as do juveniles. Finally, unrecognized genetic contributions to social recognition memory may exist between C57Bl6/J, RAG1 mice, and the C57BL/6JOlaHsd strain used in the procedures by Noack et al. (2010).

We did not observe differences between RAG1KO mice and other genotypes in rates of habituation to non-social odors or to an open-field, suggesting that the data do not likely reflect differences in general habituation to novelty. RAG1KO mice showed similar increases in investigation upon presentation of novel non-social odors, reflecting the ability to perceive and habituate to odors (Fig. 4). However, it is known that, in addition to the contribution of the main olfactory system, an additional olfactory system mediated by the accessory olfactory bulb contributes to the processing of conspecific odors in rodents (Johnston, 1985). Although there was no difference in the initial investigation duration towards the stimulus mouse in RAG1KO mice compared to the other genotypes, differences in social recognition memory performance among RAG1KO mice may reflect effects on olfactory function specific to the detection of conspecific odors. Nevertheless, the results reported here cannot rule out the possibility that olfactory-mediated processes involving RAG1 contribute to social recognition memory performance. Future studies are needed to discern the impact of RAG1-deletion on memory in the absence of social-olfactory cues.

We also did not find differences in rates of habituation in exploratory behavior or in the number of center entries in the open-field—an index of anxiety-like behavior (Post et al., 2010)—upon exposure to a novel environment (Fig. 5). These data stand in contrast to those reported by Cushman et al. (2003) who

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reported no significant habituation to an open-field over a 60 min trial. However, it must be noted that the comparison between RAG1KO and RAG1WT mice by Cushman et al. (2003) is complicated by the fact that the mice used were derived from different breeding pairs. As maternal care exerts substantial effects on behavior in adult offspring, including anxiety-like behavior (Champagne and Curley, 2009), it is possible that our use of littermates was an important control for such differences. In addition, although the number of initial total movements reported by Cushman et al. (2003) is comparable to our findings, since our testing was done under conditions similar to those used for social recognition memory behavior (i.e., the dark phase of the circadian cycle), it is also possible that circadian effects played a role in the disparities between these two studies.

At this time, the molecular mechanisms involved in RAG1 function in the CNS are unknown; however, there are two logical possibilities. First, RAG1 may participate in a somatic recombination-like process in the CNS similar to its role in the immune system. For example, candidate genes, such as the protocadherin superfamily of neural cell adhesion molecules that may specify neural circuitry during development, have been found to resemble the immunological loci in that they have multiple segments that are recombined to form various receptor mRNAs (Yagi, 2003). Olfactory receptors, taste receptors, and pheromone receptors—playing direct roles in the primary sensory modalities involved in conspecific recognition (Johnston, 1985)—have been proposed as candidates for DNA rearrangement in the brain due to their highly diversified repertoires (Yagi, 2003). However, there is as yet no evidence of altered DNA in mice cloned from individual olfactory receptor neurons (Eggan et al., 2004) nor does RAG1 appear to be involved in axonal targeting in olfactory sensory neurons or in amino acid detection (Feng et al., 2005). Nevertheless, the activities of several other molecular mechanisms, including DNA double strand break repair enzymes, are common to the immune system and the CNS (Chun and Schatz, 1999a; Chun and Schatz, 1999b). For example, pharmacological blockade of DNA ligases and polymerases with the nucleoside analog 1-beta-D-arabinofuranosylcytosine triphosphate (ara-CTP) during adulthood impairs hippocampally-mediated contextual fear memory (Colon-Cesario et al., 2006). Indeed, there is increasing evidence that a number of molecules involved in immunological function play a role in activity-dependent plasticity and brain development (Huh et al., 2000). Second, RAG1 may have a role in the CNS that is entirely distinct from somatic recombination. Evidence for this possibility comes from studies of the molecular structure of the recombinase enzymes themselves. Whereas the DNA cleavage and rearrangement of V(D)J recombination absolutely requires both RAG1 and RAG2, the RAG1 protein contains the catalytic DNA-binding core of the recombinase (Fugmann et al., 2000). Interestingly, this domain is similar to the active site of several transposases and integrases (Spanopoulou et al., 1996; Zhou et al., 2004). Kelch motifs that mediate the interaction of RAG2 with RAG1 have been observed in numerous proteins, and the discovery that a single kelch motif can mediate protein–protein interactions between RAG2 and RAG1 offers the possibility that RAG1 may interact with as yet unidentified protein(s) in the CNS (Aidinis et al., 2000; Prag and Adams, 2003).

Although the molecular mechanisms of RAG1 activity in CNS function remain to be identified, our data have implications that may extend to a broad class of neurological conditions. Mutations of RAG1 in humans lead to heterogeneous immune and clinical manifestations ranging from severe combined immunodeficiency to Omenn's syndrome (Villa et al., 2001a; Villa et al., 2001b). In the brain, RAG1 expression is upregulated in cortical dysplasia, a well-recognized cause of intractable epilepsy (Kim et al., 2003). Our results suggest that these individuals also have altered CNS function that to date has been overlooked. Future studies geared towards expanding knowledge of the consequences of RAG1-deletion for behavior and neurophysiology, identifying proteins associated with RAG1 in the brain, and RAG1's role in DNA binding in neurons will be important in elucidating the function of RAG1 in the CNS.

4. Experimental procedures

4.1. Mice

Male adult mice between 3 and 5 months of age were used as subjects in all experiments. For data shown in Fig. 1, subjects were purchased from Jackson Laboratories (RAG1KO strain was B6.129-Rag1^{tm1Mom}, stock #002096; RAG1WT strain was B6.129SF2/J, stock #101045). For data shown in Figs. 3–5, mice were purchased from Jackson Laboratories as above (RAG1KO and RAG1WT) and also from Taconic Laboratories (RAG2KO strain was B6.SJL-Ptprc^{a}/BoCrTac-Rag2^{tm1}, stock #000461-M; RAG2WT strain was B6.SJL-Ptprc^{a}/BoAiTac, stock #004007) and maintained in the pathogen-free "barrier" isolation facility of the Duke University vivarium. As shown in Fig. 2, male founders homozygous for disrupted RAG1^{null} or RAG2^{null} alleles on the C57BL/6 genetic background were crossed with C57BL/6J females to generate (RAG1^{HET}RAG2^{WT} and RAG1^{KO}RAG2^{HET}) F1 progeny. Intercrosses between unrelated F1 mice produced F2 offspring with genotypes RAG1^{HET}RAG2^{HET}, RAG1^{HET}RAG2^{WT}, RAG1^{WT}RAG2^{HET}, and RAG1^{WT}RAG2^{WT} in expected Mendelian ratios. Genotypes were determined by standard PCR methods using DNA from tail samples. F2 intercrosses of RAG1^{HET}RAG2^{HET} mice produced the F3 generation animals used to measure the longevity of social memory. This mating strategy homogenizes potential differences in the genetic backgrounds and maternal effects of the RAG1- and RAG2-deficient parental lines. RAG1KO, RAG2KO, and phenotypically normal RAG1HET and RAG2HET F3 heterozygotes were provided blindly for behavioral experiments. Stimulus C57Bl6/J female mice used for all social recognition memory experiments were purchased from Jackson Laboratories, ovariectomized at approximately 8 weeks of age, and allowed at least 1 week to recover before testing began. Mice were housed in groups of 3–5 mice per cage and were tested during the dark phase of the circadian cycle (23:00–05:00). Mice were tested and data were coded by an observer blind to genotype, and all procedures were conducted with the approval of the Duke University Institutional Animal Care and Use Committee.

4.2. Social recognition memory

Prior to social recognition testing, all subjects were exposed to the testing room and to novel stimulus mice (ovariectomized

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C57Bl6/J females) on three consecutive days to familiarize them with the room and procedure. During testing, subjects were removed from their home-cages and placed in a clean cage with fresh bedding prior to each encounter with stimulus mice. During an "initial encounter," a stimulus mouse was introduced into a test cage with the subject for 4 min. The duration of investigation, consisting of direct anogenital and face contact, pawing, climbing over, and close following (within 2 cm) was summed over the course of the trial (Gheusi et al., 1994; Kogan et al., 2000). After retention intervals of 30, 60, or 120 min, the familiar mouse and a novel stimulus mouse were introduced for a 4 min "discrimination" test, during which the investigatory behavior of the subject towards each stimulus mouse was measured. During both the initial encounter and the discrimination test, rare instances of investigation of the (male) subject by the (female) stimulus mice were excluded from measures of the duration of investigation. The order of presentation of the stimulus mouse on the initial encounter (one of the two stimulus mice used during the discrimination trial) was counterbalanced across trials. Experimentally naïve stimulus mice were used at each delay interval. Subjects that repeatedly fought with or attempted to mount the (non-receptive) stimulus mice were excluded from analysis. During testing, each subject's cage was blocked from the view of the experimenter by opaque blinds and dividers, and behavior was monitored via a video camera. To control for the transfer of odor cues between stimulus mice and subjects, the experimenter either changed gloves or sprayed his gloves with disinfectant after handling each animal. Each subject was tested 3 times at each delay interval and the median duration of investigation was used for data analysis. For experiment 2, the order of the trials (30 min, 60 min, 120 min) was pseudorandomly assigned. Data were transcribed from the video record using the Noldus Observer software, which allowed accurate recording of the duration of investigation toward the stimulus mice (Noldus Information Technology Inc., Leesburg, VA, USA). Raw data were binned into 1 min intervals using software written in visual basic by the experimenter.

4.3. *Olfactory habituation*

Subjects were allowed to habituate to a new home-cage with fresh bedding for 20 min and then olfactory stimuli were presented by dipping a cotton-tipped applicator into the stimulus solution and passing the stimuli through the wire grids of the cage. The applicator was held in place by anchoring it to a plastic weigh boat, which ensured that the stimuli were presented at a level of approximately 4.4 cm above the cage floor (Wrenn et al., 2003). The odorants used were sesame oil (control), 1% lemon scent diluted in sesame oil, and 0.1% peppermint scent diluted in sesame oil. Scents and the dilutions used were selected based upon pilot experiments showing that there was no preference for either scent. Each stimulus was presented for 3 min and then replaced with a new applicator 3 successive times, for a total of 9 presentations. Stimuli were presented in the following order: oil (3×), lemon (3×), and peppermint (3×). The experimenter viewed the interaction on a video monitor and recorded the time spent investigating the stimulus using the Noldus Observer software (Noldus Information Technology Inc., Leesburg, VA, USA). Raw data were binned into 3 min intervals using software written in visual basic by the experimenter. Investigation was defined as direct contact with the applicator, orienting towards the applicator with the head within 2 cm of it and rearing with the head oriented towards the applicator within 2 cm of it. Occasional chewing was not considered olfactory investigation (Wrenn et al., 2003).

4.4. *Open-field behavior*

Open-field behavior was assessed in 43.2 cm × 43.2 cm Plexiglas boxes equipped with an array of 16 infrared photobeams controlled by software present on a computer (ENV-515 with SOF-811 open-field activity software; Med Associates, VT, USA). For each of 3 consecutive days prior to the start of testing, mice were brought into the experimental room and handled for at least 5 min to habituate them to the experimenter and room. On the day of testing, each subject was allowed to explore the empty open-field for 60 min. Locomotor activity in the open-field (total movements) was recorded for each minute of the 60 min habituation trials. In addition, the number of entries into the center of the open-field, defined by movements more than 2.5 beams from the walls, were evaluated. Between each trial, the boxes were cleaned with 95% ethanol and allowed to air dry to attenuate and homogenize olfactory traces. Software written in visual basic by the experimenter was used to bin the data into 5 min blocks for statistical analysis.

4.5. *Statistical analysis*

Statistical analyses were done using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA, USA) or the Analysis ToolPak in Microsoft Excel 2004 for Mac (Microsoft Corporation, Redmond, WA, USA). For the social recognition memory task described in experiment 1, differences between genotypes in the duration of investigation of the stimulus mouse on the initial encounter were examined by unpaired Student's *t*-tests. For the social recognition memory tasks described in experiment 2, differences between genotypes in the duration of investigation of the stimulus mouse on the initial encounter were examined by factorial ANOVA followed by Newman–Keuls post-hoc testing. For the discrimination trials in experiments 1 and 2, data were analyzed as previously described (Engelmann et al., 1995; Noack et al., 2010) to specifically determine whether or not recognition of the previously encountered stimulus mouse had occurred. As such, differences between same versus novel stimulus mouse were examined by paired Student's *t*-test for each genotype, with increased investigation of the novel stimulus mouse relative to the same stimulus mouse taken to indicate that recognition had occurred. Standardized effect sizes for repeated measures (Cohen's *d*) were calculated according to the following formula: $d = (x1 - x2)/(s^*(\sqrt{(1-r)}))$, where x1−x2 is equal to the mean difference between investigation of the novel and familiar stimulus mouse on the discrimination trial, *s* is the pooled standard deviation, and $\sqrt{(1-r)}$ is the square root of 1 minus the correlation coefficient (Cohen, 1977). Because, with the exception of the RAG1HET mice at the 30 min interval, there were no significant differences between investigation of the novel stimulus animal and the same stimulus animal during the

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last 2 min of the 4 min discrimination trial at all delay intervals ($P>0.05$), investigation on only the first 2 min of each discrimination trial at each delay interval was subject to analysis. Data for the olfactory habituation task were analyzed as previously described (Schellinck et al., 1992) using ANOVA to assess effects of genotype and differences across trials in time spent investigating each odor. Newman–Keuls post-hoc tests were used to determine which trials differed from each other. Open-field behavior was analyzed by ANOVA with genotype as the between groups measure and time as the within groups measure. Differences between genotypes across each time block were examined using Bonferroni-corrected post-hoc comparisons. All data are presented as mean (±SEM) and data were considered statistically significant at $P<0.05$.

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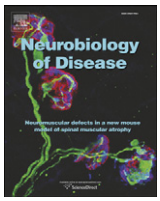

Review

The epigenetics of social adversity in early life: Implications for mental health outcomes

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ABSTRACT

An organism's behavioral and physiological and social milieu influence and are influenced by the epigenome, which is composed predominantly of chromatin and the covalent modification of DNA by methylation. Epigenetic patterns are sculpted during development to shape the diversity of gene expression programs in the organism. In contrast to the genetic sequence, which is determined by inheritance and is virtually identical in all tissues, the epigenetic pattern varies from cell type to cell type and is potentially dynamic throughout life. It is postulated here that different environmental exposures, including early parental care, could impact epigenetic patterns, with important implications for mental health in humans. Because epigenetic programming defines the state of expression of genes, epigenetic differences could have the same consequences as genetic polymorphisms. Yet in contrast to genetic sequence differences, epigenetic alterations are potentially reversible. This review will discuss basic epigenetic mechanisms and how epigenetic processes early in life might play a role in defining inter-individual trajectories of human behavior. In this regard, we will examine evidence for the possibility that epigenetic mechanisms can contribute to later-onset neurological dysfunction and disease.

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Contents

Introduction

Different cell types execute distinctive programs of gene expression that are highly responsive to developmental, physiological, pathological and environmental cues. The combination of mechanisms that confer long-term programming to genes and could bring about a change in gene function without changing gene sequence is herein termed epigenetic changes. We propose a definition of epigenetics that includes any long-term change in gene function that persists even when the initial trigger is long gone that does not involve a change in gene sequence or structure. Thus, a change in chromatin or DNA methylation in a postmitotic neuron that lasts for a long period of time would be considered an epigenetic change even in the absence of cell division. This definition stands in contrast to some classical definitions of epigenetics that require heritability in dividing somatic cells or even through germ line transmission across generations. The less strict definition of epigenetics proposed here is especially important in understanding long-term changes in gene function in the brain. Stable changes in chromatin or DNA modification in postmitotic neurons or dividing cells could be environmentally driven, may occur in response to triggers at different points in life and are potentially reversible, whereas genetic differences are germ line transmitted, virtually fixed and irreversible.

Much of the phenotypic variation seen in human populations might be caused by differences in long-term programming of gene function rather than the sequence per se, and any future study of the basis for inter-individual phenotypic diversity should consider epigenetic variations in addition to genetic sequence polymorphisms (Meaney and Szyf, 2005). In effect, epigenetic silencing and genetic silencing could have similar phenotypic consequences. Therefore, mapping the epigenome is potentially as important as the mapping of the genome in our quest to understand phenotypic differences in humans.

By extension, identifying epigenetic differences that are associated with behavioral pathologies has important implications for human health because they are potentially reversible and amenable to therapeutic intervention (Szyf, 2001; Szyf, 2009). Drugs targeting epigenetic mechanisms are currently being tested in clinical trials in psychiatric disorders (Simonini et al., 2006). It is our view that once we understand the rules through which different environmental exposures modify epigenetic processes, we may also be able to design behavioral strategies to prevent and reverse deleterious environmentally driven epigenetic alterations. The dynamic nature of epigenetic regulation, in contrast to the virtually static nature of the gene sequence, provides a mechanism for reprogramming gene function in response to changes in life style trajectories. In this way, epigenetics may provide explanations for well defined environmental effects on phenotypes. Epigenetic changes have now been associated with a number of paradigms involving social behavior in animal models—for example stress (Fuchikami et al., 2009), animal models of PTSD (Chertkow-Deutsher et al., 2009), chronic social defeat (Tsankova et al., 2006) and extinction of conditioned fear (Bredy et al., 2007). Rather than to attempt an exhaustive review of what is currently a somewhat disparate literature, our focus on early life adversity will serve as a platform to discuss the basic mechanisms involved in epigenetic programming and our studies of epigenetic differences associated with early life social adversity in humans.

The epigenome

Chromatin and the histone code

The epigenome consists of the chromatin, a protein-based structure around which the DNA is wrapped, as well as a covalent modification of the DNA itself by the methylation of cytosine rings found at CG dinucleotides (Razin, 1998). The epigenome determines the accessibility of the DNA to the transcription machinery, which converts genetic information into the messenger RNA necessary for gene function. Inaccessible genes are therefore relatively silent whereas accessible genes are actively transcribed. Densely packaged chromatin can be visualized microscopically and is termed heterochromatin while open accessible chromatin is termed euchromatin. Recently, another new level of epigenetic regulation by small non-coding RNAs termed microRNA has been discovered (Bergmann and Lane, 2003), which has already been suggested to play an important role in behavioral pathologies in humans (Vo et al., 2005), as has been reviewed elsewhere (Mehler and Mattick 2006; Mehler and Mattick, 2007; Qureshi and Mehler, 2009).

The basic building block of chromatin is the nucleosome, which is made up of an octamer of histone proteins. The N-terminal tails of these histones are extensively modified by methylation, phosphorylation, acetylation and ubiquitination. The state of modification of these tails plays an important role in defining the accessibility of the DNA wrapped around the nucleosome core. It was proposed that the amino terminal tails of H3 and H4 histones that are positively charged form tight interactions with the negatively charged DNA backbone, thus blocking the interaction of transcription factors with the DNA. Modifications of the tails neutralize the charge on the tails, thus relaxing the tight grip of the histone tails. Different histone variants, which replace the standard isoforms also play a regulatory role and serve to mark active genes in some instances (Henikoff et al., 2004). The specific pattern of histone modifications was proposed to form a "histone code," that delineates the parts of the genome to be expressed at a given point in time in a given cell type (Jenuwein and Allis, 2001).

Histone modifying enzymes and chromatin remodeling

The most investigated histone modifying enzymes are histone acetyltransferases (HAT), which acetylate histone H3 and H4 at different residues as well as other histone deacetylases (HDAC), which deacetylate histone tails (Kuo and Allis, 1998). Histone acetylation is believed to be a predominant signal for an active

chromatin configuration (Perry and Chalkley, 1982; Lee et al., 1993). Deacetylated histones are characteristic of chromatin associated with inactive genes. Histone tail acetylation is believed to enhance the accessibility of a gene to the transcription machinery whereas deacetylated tails are highly charged and believed to be tightly associated with the DNA backbone and thus limiting accessibility of genes to transcription factors (Kuo and Allis, 1998).

Some specific histone methylation events are associated with gene silencing and some with gene activation (Lachner et al., 2001). Particular factors recognize histone modifications and further stabilize an inactive state. Recently described histone demethylases remove the methylation mark causing either activation or repression of gene expression (Shi et al., 2004; Tsukada et al., 2006). Chromatin remodeling complexes, which are ATP dependent, alter the position of nucleosomes around the transcription initiation site and define accessibility of regulatory regions to the transcription machinery. It is becoming clear now that there is an interrelationship between chromatin modification and chromatin remodeling (Bultman et al., 2005).

A basic principle in epigenetic regulation is targeting. Histone modifying enzymes are generally not gene specific. Specific transcription factors and transcription repressors recruit histone-modifying enzymes to specific genes and thus define the gene-specific profile of histone modification (Jenuwein and Allis, 2001). Transcription factors and repressor recognize specific *cis*-acing sequences in genes, bind to these sequences and attract the specific chromatin modifying enzymes to these genes through protein-protein interactions. Signal transduction pathways, which are activated by cell-surface receptors, could serve as conduits for epigenetic change linking environmental triggers at cell surface receptors with gene-specific chromatin alterations, leading to the reprogramming of gene activity.

DNA methylation and gene expression silencing

The DNA molecule itself can be chemically modified by methyl residues at the 5′ position of the cytosine rings in the dinucleotide sequence CG in vertebrates (Razin, 1998) (Fig. 1). What distinguishes DNA methylation in vertebrate genomes is the fact that not all CGs are methylated in any given cell type (Razin, 1998), resulting in cell type specific patterns of methylation. Thus, the DNA methylation pattern confers upon the genome its cell type identity. Since DNA methylation is part of the chemical structure of the DNA itself, it is more stable than other epigenetic marks and thus it has extremely important diagnostic potential in humans (Beck et al., 1999).

A growing line of evidence supports the idea that, similar to chromatin modification, DNA methylation is also potentially reversible (Ramchandani et al., 1999) even in predominantly post mitotic tissues such as the brain (Weaver et al., 2004). The DNA methylation pattern is not copied by the DNA replication machinery, but by an independent enzymatic machinery (Razin and Cedar, 1977) termed DNA methyltransferase(s) (DNMTs; Fig. 2). DNA methylation patterns in vertebrates are distinguished by their correlation with chromatin structure. Active regions of the chromatin, which enable gene expression, are associated with hypomethylated DNA whereas hypermethylated DNA is packaged in inactive chromatin (Razin and Cedar, 1977; Razin, 1998).

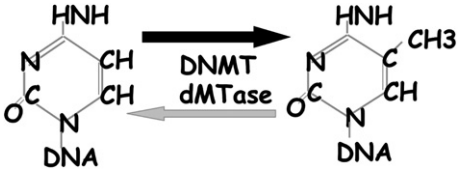

Fig. 1. The reversible DNA methylation reaction. DNA methyltransferases (DNMT) catalyze the transfer of methyl groups from the methyl donor S-adenosylmethionine to DNA releasing S-adenosylhomocysteine. Demethylases release the methyl group from methylated DNA.

DNA methylation in critical regulatory regions, including gene promoters and enhancers, serves as a signal to silence gene expression by two main mechanisms (Fig. 3). The first mechanism involves direct interference of the methyl residue with the binding of a transcription factor to its recognition element in the gene. The interaction of transcription factors with genes is required for activation of the gene; lack of binding of a transcription factor would result in silencing of gene expression. This form of inhibition of transcription by methylation requires that the methylation event occur within the recognition sequence of the transcription factor. The second mechanism is indirect. A certain density of DNA methylation in the region of the gene attracts the binding of methylated-DNA binding proteins such as MeCP2 (Nan et al., 1997). MeCP2 recruits other proteins such as SIN3A and histone modifying enzymes, which lead to the formation of a "closed" chromatin configuration and the silencing of gene expression (Nan et al., 1997). Thus, aberrant methylation will silence a gene, resulting in loss of function, which will have a similar consequence to a loss of function by genetic mechanism such as mutation, deletion or rearrangement.

Although much of our current knowledge of the role of DNA methylation in gene regulation derives from studies of effects in promoter regions, it is becoming clear that DNA methylation in other gene elements plays important roles in regulating gene function. For example, increased DNA methylation within the body of genes is typically associated with active transcription (ref). However, the function of gene body methylation remains elusive. First elucidated in plants by high-throughput sequencing methods, it has been suggested that gene body methylation might help to inhibit cryptic transcription initiation (Zilberman et al., 2007) or suppress recombination or transposon insertion within genes (Zhu, 2008). Other recent data indicate that DNA methylation at the 3′ ends of genes, as well as intragenic DNA methylation may play distinct roles on regulating gene expression (Suzuki and Bird, 2008). For example, DNA methylation within intronic regions may regulate the activity of intragenic non-coding RNAs that may be involved in regulating RNA splice variation, silencing of chromatin, degradation of mRNA and blocking translation (Mattick and Makunin, 2006). As briefly mentioned above, epigenetic regulation by small non-coding RNAs termed microRNA could potentially play an important role in behavioral pathologies in humans as well, as has been reviewed elsewhere (Mehler and Mattick 2006; Mehler and Mattick, 2007; Qureshi and Mehler, 2009).

The roles of the DNA methylation machinery and the reversibility of DNA methylation patterns

The DNA methylation machinery in vertebrates has two main roles. First, it has to establish new cell-type specific DNA methylation patterns during development and possibly during adulthood in response to new signals. Second, it has to maintain these patterns during downstream cell divisions and after DNA repair. The different enzymes and proteins of the DNA methylation machinery must address these different tasks. The methylation of DNA occurs immediately after replication by a transfer of a methyl moiety from the donor *S*-adenosyl-L-methionine (AdoMet) in a reaction catalyzed by DNMTs (Fig. 2). Three distinct phylogenic DNA methyltransferases were identified in mammals. DNMT1 shows preference for hemimethylated DNA *in vitro*, which is consistent with its role as a maintenance DNMT (Fig. 2), whereas DNMT3a and DNMT3b methylate unmethylated and methylated DNA at an equal rate which is consistent with a *de novo* DNMT role (Okano et al., 1998).

We have proposed that the DNA methylation pattern is a balance of methylation and demethylation reactions that are responsive to physiological and environmental signals and thus forms a platform for gene-environment interactions (Ramchandani et al., 1999) (Fig. 1). There are now convincing examples of active, replication independent DNA demethylation during development as well as in somatic tissues

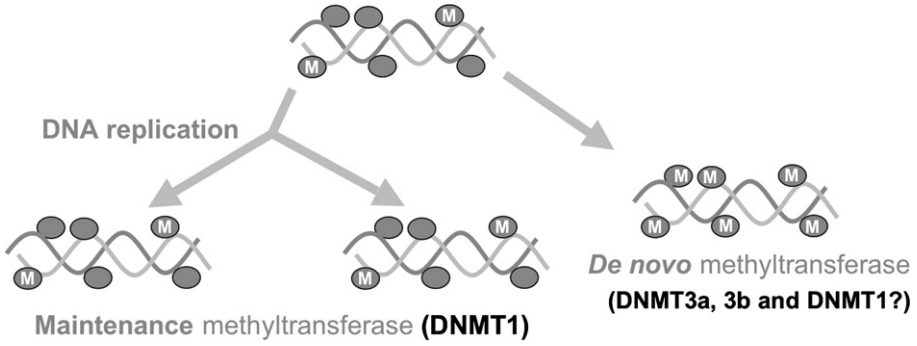

Fig. 2. DNA methylation reactions. Early after fertilization many of the methylation marks are removed by demethylases. De novo DNA methyltransferases (DNMT) add methyl groups. Once a pattern is generated it is inherited by maintenance DNMTs that copy the methylation pattern. Methyl groups are indicated by M, potential methylatable sites are indicated by an open circle.

(Lucarelli et al., 2001; Kersh et al., 2006). One example from our laboratory is that of the glucocorticoid receptor gene promoter in the brains of adult rats upon treatment with the HDAC inhibitor TSA (Weaver et al., 2004), and which has been reviewed elsewhere (e.g., see (Meaney and Szyf, 2005; McGowan and Kato, 2008a; McGowan et al., 2008b).

We also propose that the direction of the DNA methylation reaction is defined by the state of chromatin. The gene-specificity of the state of chromatin is defined by sequence-specific *trans*-acting factors that recruit chromatin-modifying enzymes to specific genes. Chromatin configuration then gates the accessibility of genes to either DNA methylation or demethylation machineries. We propose the following model: Factors that target specific chromatin modification events to genes are good candidates to define the direction of the DNA methylation equilibrium by either recruiting DNA methylation enzymes or by facilitating demethylation (D'Alessio and Szyf, 2006; Shilatifard, 2006; Berger, 2007; Suzuki and Bird, 2008).

Epigenetic contributions to mental health

Influence of DNA methylation on mental health

Genetic defects in genes encoding the DNA methylation and chromatin machinery exhibit profound effects on mental health in humans. A classic example is RETT syndrome, a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in females, which is caused by mutations in the methylated DNA binding protein MeCP2 (Amir et al., 1999). Mutations in MeCP2 and reduced MeCP2 expression have widespread neurological effects, being also associated with autism (Nagarajan et al., 2006; Ben Zeev Ghidoni, 2007; Herman et al., 2007; Lasalle, 2007). ATRX, a severe X-linked form of syndromal mental retardation associated with alpha thalassaemia (ATR-X syndrome) is caused by a mutation in a gene encoding a member of the SNF2 subgroup of a superfamily of proteins with similar ATPase and helicase domains involved in chromatin remodeling (Picketts et al., 1996). The ATRX mutation is associated with aberrant DNA methylation (Gibbons et al., 2000). Although these genetic lesions in the methylation machinery were present through development and are thus fundamentally different from methylation changes after birth, these data nevertheless support the hypothesis that DNA methylation defects could lead to mental pathologies. Thus, it is conceivable that environmental exposures affecting the activity of the methylation machinery would also lead to behavioral and mental pathologies.

There are some data indicating aberrant methylation in mental pathologies later in life in humans, although it is unclear whether these changes in DNA methylation originated during embryogenesis

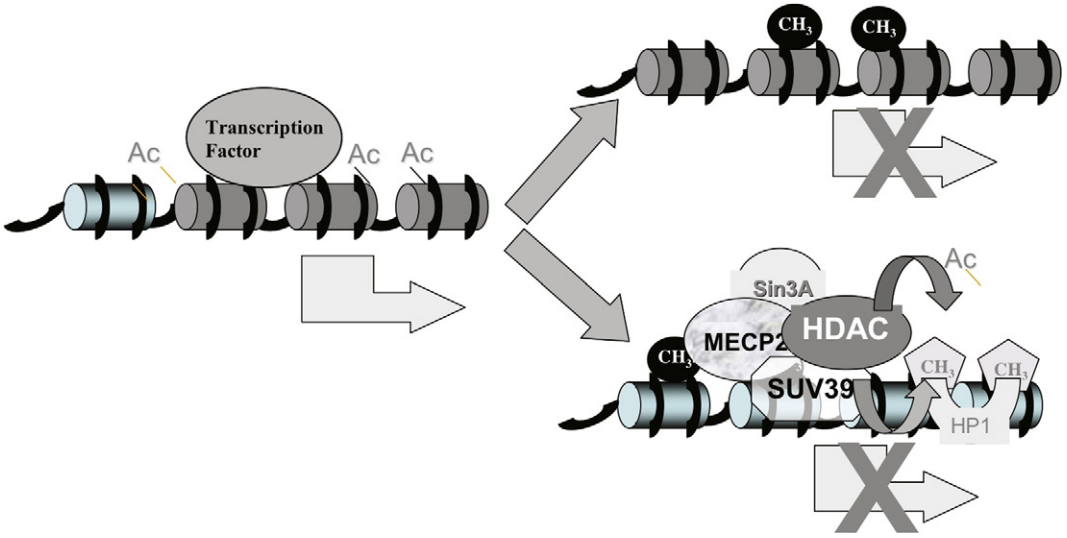

Fig. 3. Two mechanisms of silencing gene expression by DNA methylation. An expressed gene (transcription indicated by a horizontal arrow) is usually associated with acetylated histones and is unmethylated. A methylation event would lead to methylation by two different mechanisms. The methyl group (CH3) interferes with the binding of a transcription factor that is required for gene expression, resulting in blocking of transcription. The second mechanism shown in the bottom right is indirect. Methylated DNA attracts methylated DNA binding proteins such as MeCP2, which in turn recruits co-repressors such as SIN3A, histone methyltransferases such as SUV39 that methylates histones and histone deacetylases (HDAC), which remove the acetyl groups from histone tails. Methylated histones (K9 residue of histone tails) recruit heterochromatin proteins such as HP1, which contributes to a closed chromatin configuration and silencing of the gene.

or later in life as a response to an environmental exposure. For example, the gene encoding *REELIN*, a protein involved in neuronal development and synaptogenesis and implicated in long-term memory, was found to be hypermethylated in the brains of schizophrenia patients, and the methylation of the *REELIN* gene promoter was correlated with its reduced expression and increased DNMT1 expression in GABAergic interneurons in the prefrontal cortex (Chen et al., 2002; Costa et al., 2002; Costa et al., 2003; Grayson et al., 2005; Veldic et al., 2007).

Another example is the association between the DNA methylation status of the promoter of membrane-bound catechol-*O*-methyltransferase (COMT) (Abdolmaleky et al., 2006), an enzyme regulating the level of dopamine, with schizophrenia and bipolar disorder. The COMT gene has two promoters, each generating its own mRNA isoform: the membrane-bound isoform (MBCOMT) and the soluble isoform (S-COMT), respectively. One study examined the methylation status of the MB-COMT promoter in the prefrontal cortex (Brodmann's area 46) by means of a methylation-specific PCR analysis (Abdolmaleky et al., 2006). While 60% of 35 controls showed some PCR product obtained from the methylated allele, only 29% of 35 patients with bipolar disorder and 26% of 35 patients with schizophrenia showed a methylation signal. Subjects with a methylation signal showed significantly lower expression levels of MBCOMT than those not showing a methylation signal in postmortem brain samples. This study suggested the possible role of hypomethylation of the promoter of MB-COMT in both bipolar disorder and schizophrenia. These results suggest that gene-specific DNA methylation changes may be associated with increased risk of multiple forms of psychopathology.

We have described in a first published report of aberrant methylation associated with suicide that promoters of the genes encoding ribosomal RNA (rRNA) are heavily methylated in hippocampi from subjects who committed suicide relative to controls (McGowan et al., 2008c). Methylation of rRNA defines the fraction of rRNA molecules that are active in a cell, and the output of rRNA transcription defines to a large extent the protein synthesis capacity of a cell. Protein synthesis is critical for learning and memory. We found that the genetic sequence of rRNA was identical in all subjects, and there was no difference in methylation between suicide victims and controls in the cerebellum, a brain region that is not normally associated with psychopathology. These data imply that epigenetic effects that influence psychopathology likely target particular neural pathways. A reduced capacity for protein synthesis in the hippocampus of suicide victims could be epigenetically regulated, and may be involved in the pathology leading to suicide.

Thus, evidence is emerging that aberrant DNA methylation is involved in psychopathologies. Standardized forensic psychiatry methods had been used to ascertain that all of the suicide victims in our study had a history of severe abuse or neglect during childhood, which is common among suicide victims. Thus, the data suggest that severe adversity during early childhood may have been a contributing factor to the observed epigenetic pathology. It was unclear whether the observed abnormalities were a result of early adversity or whether they had emerged during adulthood as a result of the mental disorders associated with suicide. We undertook another study to address this question.

In a subsequent study, we examined the glucocorticoid receptor gene promoter in the hippocampus of human suicide victims and controls (McGowan et al., 2009). We showed previously that the epigenetic status of the glucocorticoid receptor gene promoter is regulated by parental care during early postnatal development in rats and amenable to pharmacological interventions later in life (for reviews, see (Meaney and Szyf, 2005; McGowan et al., 2008b). In our recent study, all of the suicide victims and none of the controls had a history of childhood abuse or severe neglect. A third group was composed of suicide victims with a history that was negative for childhood abuse or neglect. We found that, as in the animal model, the glucocorticoid receptor was epigenetically regulated in the human brain, and associated with altered glucocorticoid receptor gene expression. Hypermethylation of the glucocorticoid receptor gene was found among suicide victims with a history of abuse in childhood, but not among controls or suicide victims with a negative history of childhood abuse. The data are consistent with other data from the literature suggesting that suicide has a developmental origin. They suggest that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence vulnerability for psychopathology through effects on intermediate levels of function such as activity of the hypothalamic–adrenal–pituitary (HPA) axis that regulates the stress response. However, it remains unclear whether the epigenetic aberrations documented in brain pathologies were present in the germ line, whether they were introduced during embryogenesis, or whether they were truly changes occurring during early childhood.

There has been considerable interest in the DNA methylation changes in monozygotic twins discordant for mental disorders. Monozygotic twin pairs share a virtually identical genome but not the same pre- and post-natal environments, and frequently differ in their prevalence of mental disorders (e.g., see Petronis, 2006). Differences in DNA methylation were observed between monozygotic twins discordant for schizophrenia (Tsujita et al., 1998; McDonald et al., 2003; Petronis et al., 2003). Another case control analysis also found that a decreased methylation status of PPIEL (peptidylprolyl isomerase E-like) in the affected twin and in patients with bipolar II disorder was significantly correlated with its mRNA expression level, and also with the DNA methylation levels in peripheral leukocytes (Kuratomi et al., 2007). A case study of monozygotic twins discordant for Alzheimer's disease found substantially lower overall levels of DNA methylation in the temporal cortex of the affected twin (Mastroeni et al., 2009). Recently, a large-scale study of methylation discordance in monozygotic twins using whole-genome microarray methods found substantial variability across the genome in DNA methylation between twins (Kaminsky et al., 2009). These data suggest that widespread differences in DNA methylation between twins may underlie some of the variability associated with divergent incidences of mental disorders. However, caution is perhaps warranted in the interpretation of such results. For example, DNA methylation differences between monozygotic twins were reported to increase with age (Fraga et al., 2005). Consequently, it is possible that the differences in DNA methylation in discordant twins may not always be related to the pathophysiology of the illness in question.

Chromatin modifications and their roles in mental health

The fact that histone methylation is reversible provides a wide platform for pharmacological and therapeutic manipulations of the state of histone methylation in both directions. Both histone demethylases and histone methyltransferase are excellent candidates for new drug discovery. Understanding the intricate details of their genomic targets will allow the design of targeted and specific therapeutics.

The epigenetic effects of current clinically used monoamine oxidase inhibitors provide leads for the further development of therapies targeting the epigenome. For example, H3K4Me2 is a hallmark of active genes and the target of the histone demethylase LSD1, which demethylates H3-K4Me2. Interestingly, certain non-selective monoamine oxidase inhibitors used as antidepressants such as Tranylcypromine that were clinically used for some time and believed to be acting on monoamine oxidases also appear to inhibit LSD1 demethylase (Lee et al., 2006). It is possible that inhibition of LSD1 is part of the mechanism of action of these antidepressants

through activation of critical genes suppressed by the H3-K4me2 demethylating activity of LSD1 in the brain (Shi et al., 2004) or by repressing genes activated by the H3-K9Me2 demethylation activity of LSD1 (Metzger et al., 2005). Thus, it is possible that LSD1 inhibition is involved in the mechanism of action of antidepressive agents. It is tempting to speculate that selective inhibitors of LSD1 might be effective as antidepressants. This is an idea that might be pursued in the future.

Valproic acid, a long established antiepileptic and mood stabilizer, is also an HDACi (Phiel et al., 2001), suggesting a possible role for HDACi in treating mental disorders such as schizophrenia and bipolar disorder. Valproic acid has some effect in alleviating psychotic agitation as an adjunct to antipsychotics in schizophrenia (Bowden, 2007; Yoshimura et al., 2007). One recent study found that valproate, when used during pregnancy, was associated with a 6–9 point lower average IQ in offspring at 3 years of age (Meador et al., 2009). Although biological and behavioral effects of HDACi in the brain are somewhat characterized, the specific gene targets of HDACi in the brain and their function in mental pathologies are not well delineated. Nevertheless, the limited clinical data suggest a potentially important role for HDACi in treatment of mental disorders. Several clinical trials have tested valproate as an adjunctive therapy to antipsychotics in schizophrenia. There are indications that valproate might improve violent episodes in a subset of schizophrenia patients (Basan and Leucht, 2004) and might have an effect on euphoric mania in combination with antipsychotics (Bowden, 2007), as well as, features of manic symptomatology in bipolar disorders (Bowden, 2007). It should be noted that many of these trials were of small size and that further clinical trials are needed with valproate and with more potent and selective HDACi to methodically test the therapeutic potential of HDACi.

One question that needs to be addressed is whether the observed defects in histone acetylation in mental pathologies are a consequence of aberrant deregulation of the overall levels of certain HDAC isotypes or HATs, or whether it involves the aberrant targeting of HDAC to a selection of promoters. The fact that inhibition of a general enzyme such as HDAC results in exquisite positive changes in the brain implies some specificity, even for a general inhibitor of a specific class of HDACs as discussed above. It will be important to delineate the response of the transcriptomes of different brain regions to HDACi and to map the genes that are critically involved in the molecular pathology of the disease. It will also be important to characterize the critical isoforms of HDAC for regulation of these genes. The advent of isotypic specific HDACi might enhance the efficacy and potency of the treatment and reduce its toxicity.

As noted in the examples provided above, HDACi used in the treatment of psychiatric disorders, either in combination with other psychiatric drugs or alone, lack specificity for particular genes or neural networks. In much same manner as for classical drugs used in psychiatric therapy, then, it is unlikely that any epigenetic drug by itself will be entirely effective in treating mental disorders. It must also be noted that the HDACi activity of the aforementioned valproate is not the only action of this drug. As such, at present the putative direct epigenetic effects of these drugs on the symptomatology associated with mental disorders are not well understood. Thus, it will be important to develop HDACi that are specific for particular chromatin modifications as well as animal models lacking particular HDAC activities in order to directly test evidence for their molecular and behavioral mechanisms (Tsankova et al., 2007).

Summary and prospective

We propose that epigenetic mechanisms serve as an interface between the environment and the genome, and that enzymes that sculpt chromatin states and DNA methylation patterns are responsive to cellular signaling activated in the brain in response to adversity early in life. We hypothesize that the social environment early in life has a long-lasting impact on mental and physical health trajectories via epigenetic marking of specific genes. However, one important aspect of the basic epigenetic mechanisms reviewed here is that although the epigenetic markings are long-lasting they are nevertheless potentially reversible. Such data suggest, therefore, that appropriate social and pharmacological interventions could reverse deleterious epigenetic markings sculpted by negative social exposures early in life. In this review, we have focused on the epigenetic consequences of social adversity early in life and its association with clear deleterious behavioral outcomes such as suicide in humans. However, although much work remains to be done, there is some indication that positive early social experience can have a mitigating effect on stress responses later in life via epigenetic mechanisms, suggesting a protective role for positive early parental care (Weaver et al., 2004). Taken together, these data suggest that social and pharmacological interventions might activate signaling pathways in the brain that would result in a change in either the targeting or activity of the epigenetic machinery and thus a change in epigenetic markings. Epigenetic drugs are now in use in cancer and psychiatric therapy, and it is anticipated that the future will see increased use of epigenetic drugs and interventions in several other health conditions. Thus, understanding the epigenetic consequences of social exposures stands not only to revolutionize medicine but also to transform social sciences and humanities as well. Epigenetics could serve as a bridge between the social sciences and the biological sciences, allowing a truly integrated understanding of human health and behavior.

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Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse

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Maternal care influences hypothalamic-pituitary-adrenal (HPA) function in the rat through epigenetic programming of glucocorticoid receptor expression. In humans, childhood abuse alters HPA stress responses and increases the risk of suicide. We examined epigenetic differences in a neuron-specific glucocorticoid receptor (*NR3C1*) promoter between postmortem hippocampus obtained from suicide victims with a history of childhood abuse and those from either suicide victims with no childhood abuse or controls. We found decreased levels of glucocorticoid receptor mRNA, as well as mRNA transcripts bearing the glucocorticoid receptor 1_F splice variant and increased cytosine methylation of an *NR3C1* promoter. Patch-methylated *NR3C1* promoter constructs that mimicked the methylation state in samples from abused suicide victims showed decreased NGFI-A transcription factor binding and NGFI-A–inducible gene transcription. These findings translate previous results from rat to humans and suggest a common effect of parental care on the epigenetic regulation of hippocampal glucocorticoid receptor expression.

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There are maternal effects on the development of individual differences in behavioral and HPA stress responses in rodents and nonhuman primates[1,2]. Maternal behavior alters the development of HPA responses to stress in the rat through tissue-specific effects on gene transcription[3,4], including forebrain glucocorticoid receptor expression, the activation of which inhibits HPA activity through negative-feedback inhibition[5]. Thus, selective knockdown of glucocorticoid receptor expression in the corticolimbic system in rodents is associated with increased HPA activity under both basal and stressful conditions[6,7]. Conversely, glucocorticoid receptor overexpression is associated with a dampened HPA stress response[8].

Familial function and childhood adversity are linked to altered HPA stress responses in humans, which are associated with an increased risk for multiple forms of psychopathology[9–11]. There is evidence for decreased hippocampal glucocorticoid receptor expression in several psychopathological conditions associated with suicide, including schizophrenia and mood disorders[12–14]. Suicide is also strongly associated with a history of childhood abuse and neglect, and this effect is independent of that associated with psychopathology[15,16]. Thus, environmental events that associate with decreased hippocampal glucocorticoid receptor expression and increased HPA activity enhance the risk of suicide.

The effects of maternal care on hippocampal glucocorticoid receptor expression, and therefore HPA responses to stress, in the adult rodent are associated with an epigenetic modification of a neuron-specific exon 1_7 glucocorticoid receptor (*Nr3c1*) promoter[4,17]. We attempted to translate these findings to humans. We examined glucocorticoid receptor expression and *NR3C1* promoter methylation in hippocampal samples obtained from suicide victims and control subjects who died suddenly of unrelated causes. The focus of our examination was the *NR3C1* (also known as $GR1_F$) promoter, the homolog of the exon 1_7 region in the rat[18], which is highly expressed in human hippocampus[19]. Suicide victims were either positive or negative for history of childhood abuse (sexual contact, severe physical abuse and/or severe neglect), allowing for the separation of the effects associated with childhood abuse from those associated with suicide *per se*. Our controls were all negative for a history of childhood abuse.

RESULTS

Hippocampal glucocorticoid receptor expression

The human glucocorticoid receptor gene *NR3C1* covers a region of more than 80 kb in chromosome 5, containing eight coding exons (exons 2–9) and alternative 5′ noncoding exon 1s[19–21]. The 5′ untranslated region (UTR) of exon 1 of the *NR3C1* gene determines the tissue-specific expression. The 5′ UTR of *NR3C1* contains 11 exon 1 splice variants, all of which bear unique splice donor sites and share a common exon 2 splice acceptor site[19]. Exon 1_F of *NR3C1* is similar to the rat exon 1_7, which reveals a maternal effect on cytosine methylation and expression[8,18,22]. Because individuals with severe forms of major depression show decreased glucocorticoid receptor

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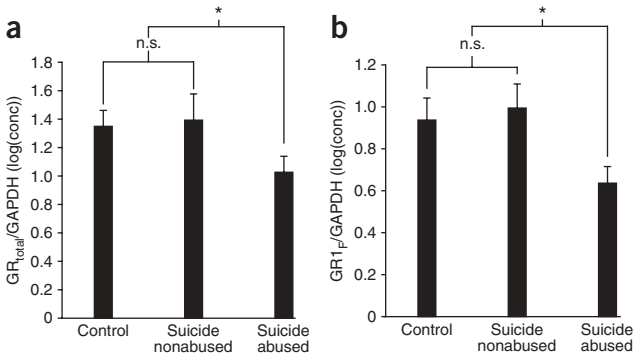

Figure 1 Hippocampal glucocorticoid receptor expression. (**a**,**b**) Mean ± s.e.m. expression levels of total glucocorticoid receptor (GR) mRNA (**a**) and glucocorticoid receptor 1_F (GR1_F) in 12 suicide victims with a history of childhood abuse, 12 nonabused suicide victims and 12 control subjects (**b**). Outliers excluded from analysis included $n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and an additional $n = 1$ suicide victim with a history of childhood abuse, and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor. * indicates $P < 0.05$; n.s. indicates not statistically significant.

expression and increased HPA activity, we hypothesized that suicide victims would show decreased expression both of glucocorticoid receptor and glucocorticoid receptor 1_F compared with control subjects.

We examined the expression of total glucocorticoid receptor and glucocorticoid receptor 1_F using quantitative reverse transcription PCR (qRT-PCR) with RNA extracted from hippocampal tissue of suicide completers with ($n = 12$) and without ($n = 12$) a history of childhood abuse and from controls ($n = 12$). There was a significant effect on glucocorticoid receptor expression ($F = 3.17$, $P = 0.05$). *Post hoc* tests showed that expression of total glucocorticoid receptor mRNA was significantly reduced in suicide victims with a history of childhood abuse relative to nonabused suicide victims or controls ($P < 0.05$); there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1a**). There was also a significant effect on the expression of transcripts containing the exon 1_F *NR3C1* promoter ($F = 3.58$, $P < 0.05$). *Post hoc* tests revealed that glucocorticoid receptor 1_F expression was significantly lower in samples from suicide victims with a history of childhood abuse compared with suicide victims without childhood abuse or controls ($P < 0.05$). Similar to the findings with total glucocorticoid receptor mRNA expression, there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1b**).

We examined the relationship between glucocorticoid receptor expression and psychiatric diagnoses (**Table 1**). Mood disorders and substance abuse disorders are risk factors for suicide and have been linked to alterations of glucocorticoid receptor expression[12]. There were no significant effects of psychopathology, even after controlling for childhood abuse status, on overall glucocorticoid receptor or glucocorticoid receptor 1_F expression ($P > 0.05$).

Genotyping and methylation analysis

Because alterations in glucocorticoid receptor 1_F activity could be derived from nucleotide sequence variation and/or epigenetic modifications, we sequenced the *NR3C1* promoter region from each subject. No sequence variation was seen among subjects and all of the sequences were identical to those published previously[19]. Moreover, for each subject, the genomic sequences targeted for binding by the primers used for bisulfite mapping were identical to the published sequence[19], thus eliminating potential primer bias between subjects in sodium bisulfite mapping.

The rat homolog of the exon 1_F *NR3C1* promoter, the exon 1_7 region, is differentially methylated as a function of variations in maternal care[4,17,22]. Cytosine methylation is a highly stable epigenetic mark that regulates gene expression via its effects on transcription factor binding[23,24]. We used sodium bisulfite mapping[25] to examine the methylation status of individual CpG dinucleotides in the *NR3C1* promoter sequence extracted from the hippocampal tissue of the same subjects used for glucocorticoid receptor expression analysis. Sodium bisulfite mapping revealed a significant effect on the percentage of methylated clones (that is, the number of clones with at least one methylated CpG site divided by the total number of clones) between groups ($F = 3.47$, $P < 0.05$). *Post hoc* tests revealed a significant difference between suicide victims with a history of childhood abuse compared with nonabused suicide victims ($P = 0.05$) or controls ($P < 0.05$). There was no difference in the percentage of methylated clones between suicide victims without childhood abuse and controls ($P > 0.05$; **Fig. 2a**). Methylation was limited to specific sites, with no clone showing global methylation (**Fig. 2b**). There were no significant correlations between levels of exon 1_F methylation and age at death ($r = 0.15$, $P > 0.05$), brain pH ($r = 0.08$, $P > 0.05$) or postmortem interval (PMI, $r = 0.24$, $P > 0.05$; **Table 1**).

Patch methylation of the *NR3C1* promoter

DNA methylation of a limited number of sites in the exon 1_F *NR3C1* promoter was associated with decreased expression of the glucocorticoid receptor 1_F variant and of total glucocorticoid receptor mRNA in suicide victims with a history of childhood abuse. Defining a causal relation between the methylation status and transcriptional efficiency of the *NR3C1* promoter is therefore of great importance. We hypothesized that DNA methylation regulates the expression of the *NR3C1* promoter through alterations in transcription factor binding. The transcription factor NGFI-A regulates the expression of *Nr3c1* promoter in the rat, an effect that is inhibited by DNA methylation[17]. To our knowledge, the regulation of NGFI-A (also known as Zif268, EGR1, Krox-24 and ZENK) has not been studied in the human hippocampus, although there is evidence that its expression is downregulated in the prefrontal cortex in schizophrenia[26]. The *NR3C1* promoter contains a number of canonical and noncanonical NGFI-A recognition elements (**Fig. 3a**). We wondered whether, as in the rat[17], NGFI-A could regulate gene transcription through the *NR3C1* promoter and whether this effect might be influenced by the methylation status of the promoter. We used a transient transfection assay in human HEK293 cells to examine transcriptional activity of a *NR3C1* promoter ligated to a promoter-less firefly luciferase expression vector (pGEM-LUC, Promega; **Fig. 3a**) in the presence or absence of ectopic NGFI-A

Table 1 Demographic characteristics and psychiatric diagnoses

	Abused suicide	Nonabused suicide	Control
Male/female	12/0	12/0	12/0
Age (years)	34.2 ± 10	33.8 ± 11	35.8 ± 12
PMI (h)	24.6 ± 5.8	39.0 ± 25.7	23.5 ± 6.0
pH	6.3 ± 0.24	6.5 ± 0.29	6.5 ± 0.22
Childhood abuse/neglect	12/0 (100%)	0/12 (0%)	0/12 (0%)
Mood disorder	8/12 (67%)	8/12 (67%)	0/12 (0%)
Alcohol/drug abuse disorder	9/12 (75%)	6/12 (50%)	5/12 (42%)

Data are presented as mean ± s.d.

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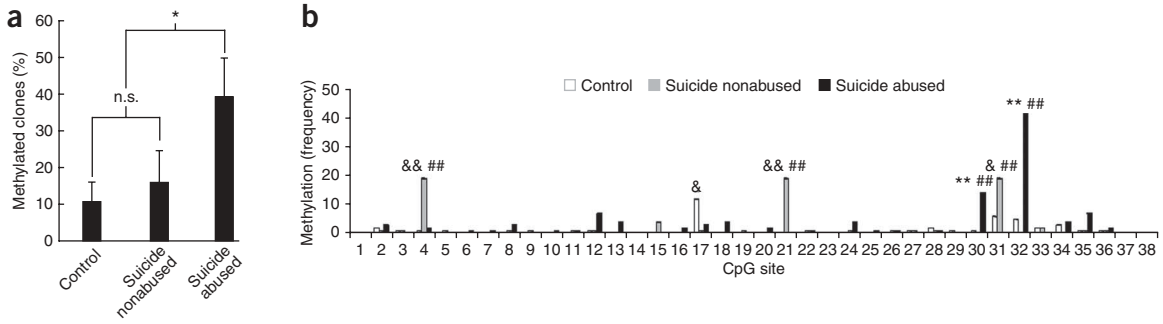

Figure 2 Methylation of the *NR3C1* promoter in the hippocampus. Twenty clones were sequenced for each subject for methylation mapping. (**a**) Mean ± s.e.m. percentage of methylated clones for suicide victims with a history of childhood abuse ($n = 12$), suicide victims without a history of childhood abuse ($n = 12$) and controls ($n = 12$). The methylation percentage was calculated as the number of clones with at least one methylated CpG site divided by the total number of clones (* indicates $P \leq 0.05$; n.s. indicates not statistically significant). (**b**) Methylation of the *NR3C1* promoter region, showing the frequency of methylation observed at each CpG site for suicide victims with a history of childhood abuse, suicide victims with no history of childhood abuse and control subjects ($^{*}P < 0.05$, $^{**}P < 0.001$, abused suicides versus controls; $^{\&}P < 0.05$, $^{\&\&}P < 0.001$, non-abused suicides versus controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, abused suicides versus non-abused suicides; Bonferroni *post hoc* comparisons).

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expression. The use of HEK293 cells allowed us to concurrently transfect a number of expression vectors with high efficiency. The absence of plasmid replication during the transient transfection assay precludes the loss of methylation via passive demethylation[27].

Luciferase expression was measured in the presence or absence of NGFI-A from the unmethylated *NR3C1* promoter plasmid compared with a methylated version. There was a significant effect of testing condition on the transcriptional activity of the exon 1_F *NR3C1* promoter ($F = 110.6$, $P < 0.0001$; **Fig. 3b**). *Post hoc* analysis revealed that the transcriptional activity of the unmethylated *NR3C1* promoter was significantly increased in the presence of the NGFI-A expression vector (*NR3C1*versus *NR3C1* + *EGR1*; $P < 0.0001$). Furthermore, methylation of the *NR3C1* promoter (the entire *NR3C1* construct was methylated *in vitro* and ligated to an unmethylated vector before transfection, *NR3C1-M*) reduced basal transcriptional activity of the *NR3C1* construct (*NR3C1* versus *NR3C1-M*, $P < 0.05$). Methylation of the *NR3C1* construct also blunted NGFI-A induction of transcription (*NR3C1* + *EGR1* versus *NR3C1-M* + *EGR1*, $P < 0.0001$).

These results indicate that methylation attenuates NGFI-A induction of gene expression through the *NR3C1* promoter. However, the decreased glucocorticoid receptor transcription observed in suicide victims with a history of childhood abuse was associated with differences in methylation levels occurring only at specific sites in the exon 1_F *NR3C1* promoter (**Fig. 2b**). An ANOVA examining the methylation of CpG dinucleotides across the exon 1_F *NR3C1* promoter revealed a significant effect of CpG site ($F = 13.86$, $P < 0.0001$), a significant effect of group ($F = 17.12$, $P < 0.0001$) and a significant interaction between CpG site and group ($F = 13.44$, $P < 0.0001$). In NGFI-A recognition elements, methylation was observed at CpG sites 12, 13, 30, 31 and 32 (**Fig. 2b**). We therefore examined whether such selective, site-specific differences in methylation could alter transcriptional activation through the *NR3C1* promoter. Two deletion constructs of the *NR3C1* promoter were generated in which selected CpG dinucleotides were patch-methylated (**Fig. 3a**). CpG sites 12 and 13 were methylated in the 255-bp construct, whereas the 125-bp promoter construct was methylated at CpG sites 30, 31 and 32. Thus, each deletion construct included at least one known or putative NGFI-A binding site[28].

We used patch methylation to examine whether selective methylation at specific sites reduces NGFI-A binding to and transactivation through the exon 1_F *NR3C1* promoter. We found an effect of methylation status on transcription factor–induced gene expression from the *NR3C1* promoter (**Fig. 3c**). For the 125-bp construct, there was a significant effect of methylation status ($F = 57.6$, $P < 0.0001$) and

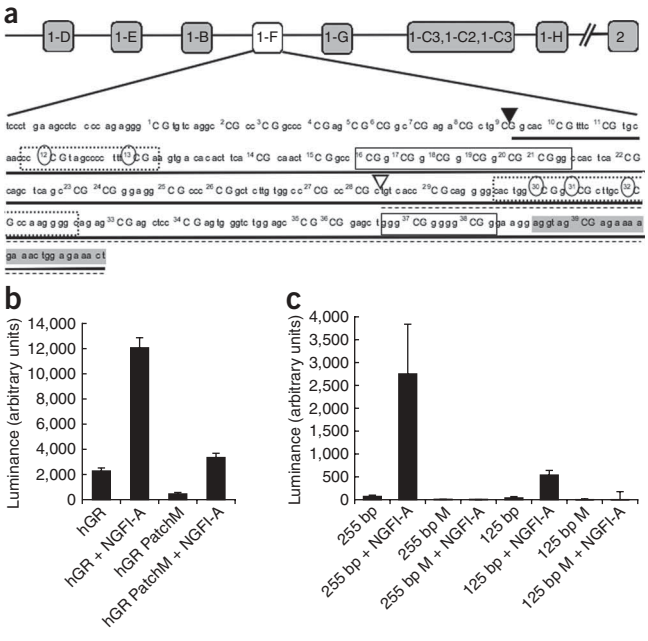

Figure 3 *In vitro* analysis of *NR3C1* promoter methylation. (**a**) The relative position of the *NR3C1* variant and the promoter sequence, showing the location of the CpG dinucleotides. The 255-bp (▼, solid underline) and 125-bp (▽, broken underline) deletion constructs are shown, along with specific CpG dinucleotides that were methylated in each deletion construct, as indicated by circles. Boxes represent known or putative canonical (solid-lined box) and noncanonical (broken-lined box) NGFI-A–binding sites, with the shaded area indicating the beginning of the exon. (**b**,**c**) Mean ± s.e.m. levels of luciferase expression in HEK293 cells. Results are shown after the subtraction of expression of the promoter in the antisense orientation. (**b**) The full *NR3C1* promoter was either unmethylated (hGR) or completely patch methylated (hGR PatchM) and transfected in the presence or absence of NGFI-A. (**c**) The 255-bp and 125-bp *NR3C1* deletion construct were either unmethylated (255 bp or 125 bp) or methylated (255 bp M or 125 bp M), as shown in **a**, and transfected either in the presence or absence of NGFI-A.

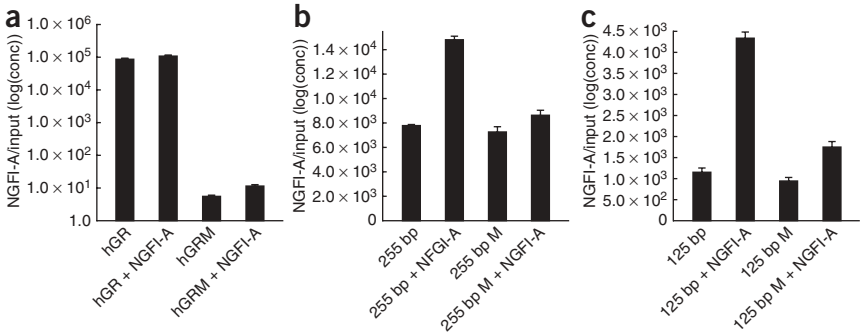

Figure 4 NGFI-A association with exon 1_F *NR3C1* promoter constructs. (**a**–**c**) Quantification of *NR3C1* promoter immunoprecipitated with NGFI-A antibody and normalized to input DNA for the full human *NR3C1* promoter (**a**), the 255-bp deletion construct (**b**) and the 125-bp deletion construct (**c**), each of which was either unmethylated or methylated and transfected in the presence or absence of NGFI-A. Data are presented as means ± s.e.m.

NGFI-A treatment ($F = 6.3$, $P < 0.05$). As predicted, there was also a significant interaction between methylation status and the NGFI-A expression ($F = 48.7$, $P < 0.0001$). *Post hoc* analysis of the 125-bp *NR3C1* promoter construct revealed that the effect of NGFI-A on gene transcription was significantly ($P < 0.001$) greater in the presence of the unmethylated rather than the patch-methylated *NR3C1* promoter construct. The same pattern of results was observed for the 255-bp *NR3C1* promoter construct. Thus, for the 255-bp *NR3C1* promoter construct, there was a significant effect on transcriptional activity of methylation status ($F = 555.4$, $P < 0.0001$) and NGFI-A expression ($F = 387.3$, $P < 0.0001$). There was also a significant interaction between methylation status and the presence of NGFI-A ($F = 489.1$, $P < 0.0001$). *Post hoc* analysis revealed a significantly ($P < 0.001$) greater effect of NGFI-A on gene transcription in the presence of the unmethylated compared with the patch-methylated 255-bp *NR3C1* promoter construct.

NGFI-A association with the *NR3C1* promoter

Site-selective differences in methylation of CpG regions in promoters can alter gene transcription by affecting transcription factor binding[24]. We carried out chromatin immunoprecipitation assays on samples that were obtained using the transfection model described above to examine the association of NGFI-A with the methylated and the nonmethylated *NR3C1* promoter constructs. The results revealed a significant effect of methylation status on NGFI-A association with the exon 1_F *NR3C1* promoter constructs transfected with *EGR1* ($F = 242.92$, $P < 0.0001$; **Fig. 4a**). *Post hoc* tests showed that, in comparison with the nonmethylated construct, patch-methylation inhibited NGFI-A binding to the exon 1_F *NR3C1* promoter ($P < 0.005$; **Fig. 4a**). For the 125-bp promoter construct, there was a significant effect of methylation status ($F = 102.28$, $P < 0.001$) and NGFI-A treatment ($F = 209.99$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 72.71$, $P < 0.005$). Similarly, for the 255-bp promoter construct, there was a significant effect of methylation status ($F = 95.18$, $P < 0.001$) and NGFI-A treatment ($F = 152.13$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 67.75$, $P < 0.005$). *Post hoc* testing revealed that, in comparison with the nonmethylated version, patch-methylation inhibited NGFI-A binding to either the 255-bp or 125-bp promoter constructs ($P < 0.01$; **Fig. 4b**,**c**). These findings suggest that the site-selective methylation of the exon 1_F *NR3C1* promoter construct, mimicking the differences observed between samples obtained from suicide victims with a history of childhood abuse, reduces NGFI-A binding and transcriptional activation through the exon 1_F *NR3C1* promoter.

DISCUSSION

Our findings indicate that hippocampal *NR3C1* gene expression is decreased in samples from suicide victims with a history of childhood abuse compared with controls (victims of sudden, accidental death with no history of abuse). In contrast, we found no differences in glucocorticoid receptor expression between suicide victims without a history of childhood abuse and controls. The pattern of results for hippocampal expression of the glucocorticoid receptor 1_F variant was identical to that of total glucocorticoid receptor expression. Our findings suggest that changes in glucocorticoid receptor expression are closely associated with a developmental history of familial adversity, in this case a history of childhood abuse, than with suicide completion. These results are also similar to those of earlier reports in which childhood abuse was associated with an increase in pituitary adrenocorticotropic hormone (ACTH) responses to stress among individuals with or without concurrent major depression[11]. These findings are particularly relevant, as pituitary ACTH directly reflects central activation of the HPA stress response and hippocampal glucocorticoid receptor activation dampens HPA activity.

Our findings are also consistent with those from studies with rodent and nonhuman primates showing that persistent disruptions of mother-infant interactions are associated with increased hypothalamic corticotrophin-releasing hormone expression and increased HPA responses to stress[1,2,29]. Variations in maternal care in the rat influence hippocampal glucocorticoid receptor expression, as well as methylation of the rat fetal calf serum *Nr3c1* promoter, the homolog of the human exon 1_F *NR3C1* promoter[3,4,17,22]. Hippocampal samples from suicide victims showed increased methylation of the exon 1_F *NR3C1* promoter in comparison with samples from controls, but only in cases with a history of childhood abuse. Neither hippocampal glucocorticoid receptor expression nor the methylation status of the exon 1_F *NR3C1* promoter was altered in suicide victims with no history of abuse. These findings suggest that variation in the methylation status of the exon 1_F *NR3C1* promoter, similar to that for glucocorticoid receptor 1_F and total glucocorticoid receptor mRNA expression, associates with childhood adversity and not with suicide *per se*.

A recent study of human cord blood found a correlation between maternal mood and neonatal methylation status of glucocorticoid receptor 1_F[30]. This study reported that increased site-specific methylation of an NGFI-A response element of glucocorticoid receptor 1_F is linked to an enhanced cortisol stress response in infants. Maternal mood disorders are associated with decreased maternal sensitivity and impaired mother-infant interactions[31], as well as with an increased risk for depression in the offspring[32]. Decreased hippocampal glucocorticoid receptor expression associates with depression[12], and psychotic and severe forms of depression are commonly associated with increased HPA activity[13,33]. Thus, our findings suggest that the transmission of vulnerability for depression from parent to offspring could occur, in part, through the epigenetic modification of genomic regions that are implicated in the regulation of stress responses.

One limitation of our design is the absence of samples from control subjects with a history of child abuse. Notably, child abuse predicts ACTH responses to stress[11]. However, the best predictor of HPA

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responses to stress is the interaction between a developmental history of child abuse and stress in adulthood. One interpretation for such findings is that childhood adversity might alter the development of systems that serve to regulate stress responses, such as hippocampal glucocorticoid receptor expression, and thus enhance the effect of stress in adulthood and vulnerability for mood disorders[11]. Rodent models provide evidence for a direct effect of variations in forebrain glucocorticoid receptor expression and the behavioral characteristics of depression. Mice bearing a brain-specific glucocorticoid receptor knockdown show behavioral alterations that mimic some of the features of depression[6,7,34].

The data reported here are consistent with previous reports of alterations in cytosine methylation associated with psychopathology[35–37]. We found increased site-specific methylation of the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse (**Fig. 2**). Our transfection studies with constructs that replicated the *in vivo* methylation profiles indicated that there was a relationship between cytosine methylation, transcription factor binding and gene expression. Variations in maternal care in the rat produce differential methylation of the exon 1_7 *Nr3c1* promoter, the rat homolog of the exon 1_F *NR3C1* promoter, which regulates hippocampal glucocorticoid receptor expression[4,17,22] and HPA responses to stress[3,4]. Increased CpG methylation of the *Nr3c1* promoter decreased NGFI-A binding and reduced hippocampal glucocorticoid receptor expression. Manipulations that reversed the differences in exon 1_7 methylation eliminated the maternal effect on NGFI-A binding, glucocorticoid receptor expression and HPA activity[4,22]. Likewise, our *in vitro* data reveal that differential methylation of the human *NR3C1* promoter altered NGFI-A binding and NGFI-A–induced gene transcription. These findings suggest that selective differences in methylation status at certain sites affect transcription factor binding and gene expression.

Because cytosine methylation is a highly stable, the differences in CpG methylation are unlikely to be a consequence of events immediately preceding death or during the postmortem period. Therefore, changes in brain pH do not affect DNA methylation[38]. The PMI did not differ between the groups and was uncorrelated with the methylation of the *NR3C1* promoter. The intermittent pattern of methylation and the fact that only a portion of the neuronal population was methylated in each subject is consistent with a model in which alterations in methylation occur at later stages in development, after the completion of embryogenesis and neuronal differentiation. Indeed, the maternal effect on the methylation status of the exon 1_7 *Nr3c1* promoter in the rat occurs during early postnatal life[4].

There are precedents for the apparent developmental origins for the observed differences in DNA methylation and glucocorticoid receptor expression. Childhood abuse in humans is associated with altered hippocampal development[39], enhanced HPA activity[9,11] and an increased risk for psychopathology[15,16]. Similarly, children exposed to childhood adversity are more likely to engage in suicidal behavior[40,41]. Variations in the parental care of children are linked with individual differences in HPA and sympathetic and central catecholamine responses to stress[1,11,42,43]. Interventions that target parental care of high-risk children alter HPA activity[44]. Thus, it is tempting to speculate that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence the vulnerability for psychopathology through effects on intermediate levels of function, such as HPA activity.

In summary, our data reveal increased site-specific methylation in the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse and suggest that there is a relationship between cytosine methylation, transcription factor binding and gene expression. Our results are consistent with evidence from studies using psychological autopsy methods[40] and epidemiological longitudinal designs[45], which suggest that suicide has a developmental origin. We acknowledge that such conclusions are based on samples that differ along a wide range of experiential and potentially genetic dimensions. Our data certainly do not exclude alternative mechanisms of vulnerability. Indeed, the challenge for the future is to understand how epigenetic variation overlaying that occurring in nucleotide sequence might explain the developmental origins of vulnerability for chronic illness. Our data are merely consistent with observations from animal studies investigating epigenetic regulation of the *Nr3c1* gene and with the hypothesis that early life events can alter the epigenetic state of relevant genomic regions, the expression of which may contribute to individual differences in the risk for psychopathology.

METHODS

Postmortem sample preparation and subject characteristics. We used hippocampal samples from the Quebec Suicide Brain Bank (http://www.douglasrecherche.qc.ca/suicide), which included 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse (matched for psychiatric diagnoses) and 12 controls. All subjects were matched for PMI, gender and age (**Table 1**). Samples were processed as described previously[46] and consisted of hippocampus tissue from male suicides and control subjects of French-Canadian origin, dissected at 4 °C and stored at –80 °C. All subjects died suddenly with no medical or paramedical intervention. Suicides were determined by the Quebec Coroner's Office and the control subjects were individuals who had died suddenly from causes other than suicide. Homogenates of tissue samples were used for genomic DNA (DNeasy, Qiagen) and RNA (Trizol, Invitrogen) extraction. Possible confounds that were examined included PMI, brain pH and the age of the donor at death. Samples were processed and analyzed blindly with respect to demographic and diagnostic variables. Signed informed consent was obtained from next of kin.

Psychological autopsies. Psychiatric diagnoses were obtained using Structured Clinical Interviews for DSM-III-R I (ref. 47) adapted for psychological autopsies, which is a validated method for reconstructing psychiatric history by means of extensive proxy-based interviews, as described elsewhere[48]. History of severe childhood sexual and/or physical abuse or severe neglect was determined by means of structured interviews using the Childhood Experience of Care and Abuse[49] questionnaire adapted for psychological autopsies[45].

Genotyping. Genomic DNA was extracted (DNeasy, Qiagen) according to the manufacturer's protocol. For PCR, we used a 5′-GGG TTC TGC TTT GCA ACT TC-3′ sense primer and a 5′-CCT TTT TCC TGG GGA GTT G-3′ antisense primer that were directed to the *NR3C1* promoter (Genebank accession number AY436590). Primers were selected that covered a 536-bp region that included the region for sodium bisulfite analyses. The resulting PCR products for each subject were sequenced bidirectionally using the forward and the reverse primer on an ABI 3100 genetic analyzer (Applied Biosystems) following the manufacturer's instructions. Genetic variation was assessed throughout the *NR3C1* promoter region used for bisulfite analysis by alignment of genomic DNA with the previously published *NR3C1* promoter sequence[19] using freely available software (CLC Workbench, CLC bio).

Methylation mapping and expression analyses. Sodium bisulfite mapping was performed as previously described[25,50] for 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse and 12 controls. Individual clones were extracted and sequenced (Cequation (8800), Beckman-Coulter). We obtained 20 clones per subject for sequencing from 2–3 independent PCR reactions.

RNA extraction was performed using Trizol (Invitrogen) and was followed by Dnase I treatment, and cDNA conversion was performed using oligo(dT) primers (Invitrogen) according to manufacturers instructions (Roche Molecular Biochemicals). The same subjects used for methylation analysis were studied

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for expression analyses using quantitative RT-PCR. Outliers with expression values that differed more than 1.5 s.d. from the mean were excluded from analysis ($n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and $n = 2$ control subjects, $n = 2$ suicide victims with a history of childhood abuse and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor; see **Supplementary Methods** online).

HEK293 cell cultures and transient transfection assays. To prepare and transfect (and see ref. 17) the unmethylated and methylated plasmids, we subjected the exon 1_F *NR3C1* promoter to PCR amplification and cloned the resulting PCR product into a PCR2.1 plasmid (Original TA cloning kit, Invitrogen). The *NR3C1* promoter was then ligated into the PCR2.1 plasmid. For patch methylation, the glucocorticoid receptor 1_F plasmid was incubated (2 h, 37 °C) with SssI DNA methyltransferase (20 U, New England Biolabs) in a buffer containing S-adenosylmethionine, and this procedure was repeated until full protection from *Hpa*II digestion was observed. Following digestion with *Hin*dIII and EcoRV restriction enzymes, each fragment was then ligated into a pGEM-luc vector (Promega) at the *Hin*dIII and *Bam*HI or XbaI and *Bam*HI sites in the 5′ to 3′ (sense) or 3′ to 5′ (antisense) orientation, respectively. The concentration of each ligation product was determined by fractionation on a 1.5% agarose gel, by comparing bands of the expected ligation product size against a standard curve of known DNA concentration (ten fivefold serial dilutions of 2 $\mu g/\mu l^{-1}$ micrococal nuclease DNA) to control for possible unequal efficiency of ligation and to ensure that equal amounts of correctly ligated plasmids were used in the transfections. The ligated plasmid was directly transfected into HEK293 cells and was not subcloned to avoid loss of methyl groups from CG dinucleotides during growth in *E. coli*, which do not express CG DNA methyltransferases. For deletion constructs of the exon 1_F *NR3C1* promoter plasmids were prepared and ligations verified in the same manner as described above, except that oligonucleotides for *NR3C1* promoter amplification were designed that incorporated *Hin*dIII and EcoRV restriction sites, obviating the need for ligation into PCR2.1 vector before ligation into the pGEM-luc vector (also see **Supplementary Methods**).

For the NGFI-A plasmid, we subcloned the *EGR1* coding sequence into a TOPO–His expression vector (pEF6/V5-His TOPO TA Expression kit, Invitrogen)[4]. In co-transfection studies, human embryonic kidney HEK293 cells were plated at a density of 6×10^4 in six-well dishes and transiently co-transfected with a total amount of 1.5 μg of plasmid DNA (1.0 μg of *NR3C1* promoter ligated into the pGEM-luc plasmid and 0.5 μg of NGFI-A expression plasmid or 0.5 μg of control pEF6/V5 plasmid) using the calcium phosphate method. HEK293 cells were maintained as a monolayer in DMEM (Invitrogen) containing 10% fetal calf serum (Colorado Serum Company). The cells were harvested 72 h after transfection and lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

Chromatin immunoprecipitation assay for NGFI-A. We carried out chromatin immunoprecipitation assays[30] by postfixing cells in 37% formaldehyde and then pelleted them before lysis and sonication. We reserved one tenth of the sample as 'input' to quantify the amount of DNA before immunoprecipitation. For the remainder of each sample, extracted chomatin was immunoprecipitated using rabbit polyclonal antibody to NGFI-A (antibody) or normal rabbit IgG (nonspecific; both from Santa Cruz Biotechnology). All of the DNA was then uncrosslinked and subjected to qRT-PCR, using primers directed at the luciferase gene immediately downstream of the transfected *NR3C1* promoter (sense, 5′-AGA GAT ACG CCC TGG TTC C-3′; antisense, 5′-CCA ACA CCG GCA TAA AGA A-3′; $T_m = 54$ °C). The signal for each sample was calculated by dividing the value of the antibody by the input. Each resulting value was multiplied by a constant (1×10^6) to plot the values obtained from the experiments on logarithmic axes.

Statistical analyses. Statistical analyses were conducted using Statview or JMP 7 (SAS Institute). Data are presented as mean ± s.e.m. For DNA methylation analysis, the percentage of methylated clones for each subject was tabulated by dividing the number of clones with at least one methylated CpG site by the total number of clones. A factorial ANOVA was used to compare the percentage of methylated clones for each subject as the dependent variable and group (suicide abused, suicide nonabused or control) as the between groups factor. To examine differential methylation across CpG sites methylated *in vitro*, we compared the total number of methylated CpG sites across the *NR3C1* promoter ($n = 33$) for all clones per group (that is, 12 subjects × 20 clones = 240 clones per group) using ANOVA followed by Bonferroni corrected *post hoc* comparisons. For RNA expression analysis, ANOVA followed by PLSD *post hoc* tests were used to examine differences between the suicide victim and control group. Unpaired *t* tests were used to compare groups of subjects with different clinical diagnoses (for example, subjects with mood disorders versus subjects without mood disorders). The relationships between DNA methylation, expression, age at death, PMI and brain pH were analyzed using linear regression analysis. Factorial ANOVA were used for *in vitro* analyses of *NR3C1* promoter, followed by *post hoc* analyses. Statistical significance was determined at $P \leq 0.05$ and analyses included type 1 error correction for multiple comparisons where applicable.

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

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Short communication

The effects of pH on DNA methylation state: *In vitro* and post-mortem brain studies

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ABSTRACT

Assessment of methylation state of DNA extracted from brain is becoming one of the most investigated issues in the study of epigenetics and psychopathology. pH effects in brain are known to affect gene transcription, though pH effects on DNA methylation state are unknown. We demonstrate *in vitro* using an artificially methylated plasmid that DNA methylation state remains stable, even under extreme pH conditions. Next, using two different genomic regions from human DNA, we assess methylation state from both cortical and sub-cortical brain regions using subjects with varying pH levels. No correlation was found between DNA methylation state and pH. These results suggest that DNA methylation state is stable in post-mortem brain.

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1. Introduction

A wealth of epigenetic studies in brain is currently under way (Abdolmaleky et al., 2004; Mill and Petronis, 2007), and methylation is one epigenetic mechanism that affects gene transcription which could mediate the interaction of genes and environment (Weaver et al., 2004). Methylation refers to the process of the addition of a methyl group to DNA and a number of proteins are known that bind methylated DNA or that add methyl groups to DNA. These protein/DNA interactions can have important repercussions on gene expression (Amir et al., 1999).

Of particular interest to psychopathological research is the methylation status of DNA isolated from post-mortem brain, but a better understanding of the potential effect of confounding factors, such as pH, is needed before associations between methylation state and certain illnesses are made. pH effects in post-mortem brain are a major caveat of gene expression studies (Vawter et al., 2006), though the effect of pH on methylation state of DNA extracted from post-mortem brain is unknown. It is possible that DNA exposed to more acidic conditions even in the absence of any biological function, could affect DNA methylation state.

This study addresses the effects of pH in post-mortem brain on DNA methylation state. Using both *in vitro* and post-mortem brain experiments, we find that DNA methylation state is stable in post-mortem brain.

2. Materials and methods

2.1. In vitro analysis of pH effects on DNA methylation

We treated the pGL3 plasmid (Promega) with SSSI methyltransferase, an enzyme that methylates all cytosine nucleotides in a CpG dinucleotide. To ensure that this step was effective, we took two sub-samples of the methylated plasmid (pGL3-CH3) solution and exposed them to two restriction enzyme digestions: HPAII and MSPI. Both of these enzymes recognize the same site (CCGG), but HPAII is blocked from cutting DNA when the internal C is methylated. The pGL3 plasmid is 4800 bp long and has 25 CCGG sites. MSPI is insensitive to the methylation status of the internal C. The *in vitro* methylated pGL3 was purified by standard phenol–chloroform extraction procedures.

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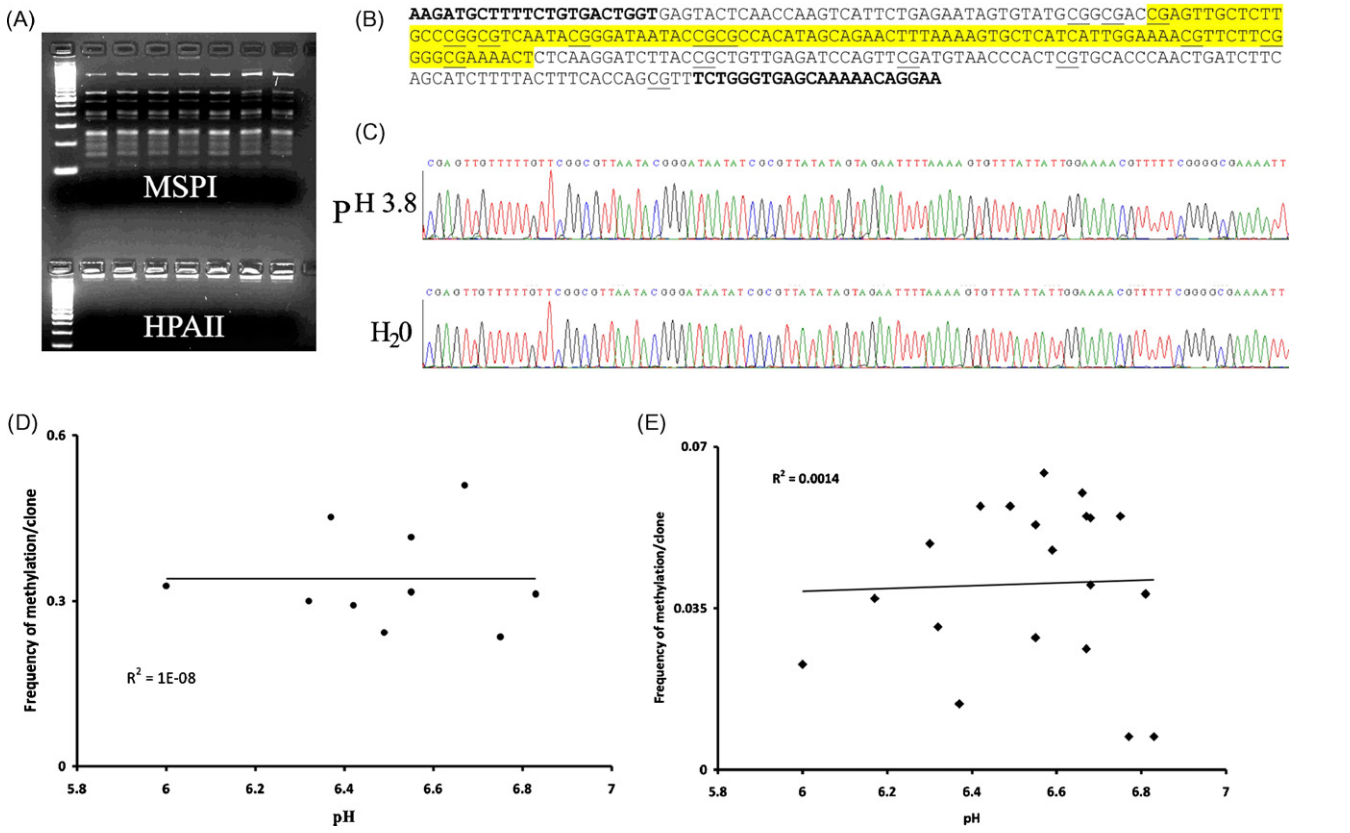

Fig. 1. pH does not affect methylation status of DNA *in vitro* or in post-mortem tissue. (A) Successful methylation of the pGL3 plasmid. (B) Tested sequence from pGL3 plasmid for the described experiment. pGL3 primers specific for bisulfite-treated plasmid are emboldened. CpG sites are underlined. Yellow highlight demonstrates area where sequence traces in 1C are taken. (C) Sequences from pure water and pH 3.8. Note that all CpG sites are methylated and C sites not in a CpG dinucleotide are detected as thymidine. (D) pH versus frequency of methylation, rRNA, and (E) NTRK2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Following ethanol precipitation the plasmid DNA was re-suspended and incubated for 48 h at 22 °C in pure water and a series of solutions that differed in pH (3.8, 6.1, 6.62, 7.2, 10 and 12). pH solutions were made using HPLC-grade water with NaOH and HCl. Following incubation, the DNA samples were treated with sodium bisulfite following the manufacturer's protocol (Qiagen EpiTec Bisulfite Kit).

Bisulfite treatment converts all cytosine residues to uracil, but methylated cytosines remain intact (Clark et al., 1994). This treatment creates a sequence difference between methylated and unmethylated cytosines which enables mapping of the methylation pattern at single base resolution. Primers specific for pGL3-CH3 were designed using Methyl Primer Express (Fig. 1B; forward: 5′-AAGATGTTTTTTTGTGATTGGT-3′; reverse: 5′-TTCCTATTTTTACTCACCCAAA-3′). Using these primers in a PCR reaction, a product of 278 bp was generated.

2.2. pH measurements from human brain

We followed the protocol used by Vawter et al. (2006) to take pH measurements. 80–120 mg of cerebellar tissue was homogenized in Chromosolv water (ultrapure water normally used for high performance liquid chromatography—Sigma–Aldrich) at a 10:1 water to tissue ratio. All tissue was taken from previously frozen brains. Tissue was homogenized with a TissueTearor (Biospec Productions Inc.), on ice, until no brain fragments were visible. After re-equilibration to room temperature, solutions were measured with a Corning pH meter.

2.3. Subjects

All subjects in this study were recruited at the Montreal Morgue as part of on-going recruitment of subjects for the Douglas Hospital Brain Bank. All subjects were male and did not die in an extreme agonal state, according to medical charts and/or informant reports. After death and permission from next-of-kin, brains were extracted, sectioned based on Brodmann region at 4 °C and snap frozen in isopentene at −80 °C. Brains were then stored at −80 °C. DNA was extracted from the dorsolateral prefrontal cortex (BA 9) and hippocampus from each subject and bisulfite treated.

2.4. Post-mortem analysis of pH effects on DNA methylation state

Two different primer pairs were used to assess methylation status in CpG rich promoter regions: ribosomal RNA gene regulatory region (U13369)—forward: 5′-GTT TTT GGG TTG ATT AGA-3′; reverse: 5′-AAA ACC CAA CCT CTC C-3′. DNA used was from hippocampus. NTRK2 promoter (NM_000346)—forward: 5′-GAGAGTGGGTATATTGGTGGTTTTA-3′; reverse, 5′-CCAACTTATCAAAAACTAAACTAATCC-3′. DNA used was from BA 9. The amplified products were extracted from the gel, ligated into a pDrive vector, and transformed into competent *Escherichia coli* cells (Qiagen PCR Cloning*Plus* Kit). Incorporation of the correct DNA fragment was verified by restriction enzyme digestion. All sequencing was done at the Genome Quebec Innovation Centre. At least 8 clones were used for each subject and for each primer pair.

3. Results

To test the effects of pH *in vitro* we first needed a fully methylated DNA sequence with which to perform the experiment. We selected the pGL3 plasmid due to plasmid availability and presence of a number of CpG dinucleotides inside of an easily amplifiable region. We first exposed the plasmid to SSSI methyltransferase to methylate all CpG dinulceotides. To test the effectiveness of this step, we exposed the experimentally methylated plasmid (pGL3-CH3) to two restriction enzymes: The first (HPAII), an enzyme incapable of cleaving methylated CpG dinucleotides and the second (MSPI), an enzyme fully capable of cleaving methylated CpG dinucleotides. Both enzymes recognize CCGG site for cleavage. Fig. 1A demonstrates the resulting gel from the pGL3-CH3 plasmid being treated with each of the two enzymes.

We next used the pGL3-CH3 plasmid to assess the effects of pH on DNA methylation patterns *in vitro*. We made a wide range of pH solutions (3.8, 6.1, 6.62, 7.2, 10 and 12) and incubated pGL3-CH3 for 48 h with differing pH solutions and pure water. After incubation, we extracted pGL3-CH3 from the solutions, bisulfite treated the extract, and amplified a small region of DNA within the plasmid. The amplified product was then cloned into a pDrive vector (at least 8 clones per solution) and sequenced. We found that the sequences from plasmids incubated in varying pH solutions were indistinguishable from those incubated in pure water (Fig. 1B and C). All cytosines residing in the dinucleotide CpG sequence remained methylated under all conditions while all cytosines found in other sequence contexts were detected as thymidine bases, indicating lack of methylation.

We next tested the effects of pH on DNA methylation status in post-mortem brain. All subjects used in this study underwent full psychological autopsy procedures at the McGill Group for Suicide Studies (Dumais et al., 2005). We used two different primer pairs and two different brain regions for this study.

We first analysed ribosomal RNA (rRNA), a gene known to have a heavily methylated promoter region (Ghoshal et al., 2004). After sequencing clones from 10 individuals using DNA extracted from hippocampus, we found no significant correlation between pH and methylation state of the rRNA gene (Fig. 1D; PCR product size of 250 bp with 27 CpG dinucleotides). We next analysed the promoter of a gene that has been investigated as a candidate in psychiatric disorders (NTRK2 (Dwivedi et al., 2003)) in 20 subjects (10 of whom were the same as for the rRNA analysis) in frontal cortex. No correlation was found between pH and methylation state (Fig. 1E; PCR product size of 440 bp with 35 potential CpG dinucleotides).

4. Discussion

This study has demonstrated that DNA methylation state is a stable phenomenon, at least in regards to acidity and alkalinity in post-mortem brain and *in vitro*. We first demonstrated this under extreme pH conditions (i.e. pH conditions outside of physiological range for human brain) *in vitro* and then under physiological pH conditions in post-mortem brain tissue.

This study did not investigate *in vivo* effects of pH on DNA methylation. The purpose of this study was to understand whether pH in post-mortem tissue is a relevant confounding factor in experiments where DNA is extracted from brain to be used for methylation analyses. If DNA methylation is an active process, as has been suggested (Kangaspeska et al., 2008; Metivier et al., 2008), than pH changes may affect DNA methylation *in vivo*.

One time point of interest that could not be addressed by this study is the point from death to brain preservation in cold storage, i.e. methylation state changes during the post-mortem interval. Immediately after death, some brain cells are still alive and lactic acidosis may occur (Alafuzoff and Winblad, 1993; Ravid et al., 1992). This potential change in pH as brain cells die is accompanied by a host of other physiological conditions (e.g. hypoxia, apoptosis and necrosis) each of which is a variable that could alter DNA methylation state. Studying only pH effects on DNA methylation status during brain death, even in a controlled laboratory setting using animals, is technically very challenging.

What this study can conclude, however, is that once pH is set (once the brain is in cold storage) there is no correlation across subjects between pH and methylation state of DNA. This could be directly assessed using DNA incubated in solutions that differed in pH. This allowed the examination of pH effects on DNA methylation state without the interference of any other biological factors. We caution that our post-mortem correlation results apply only to the range of pH reported in this study (6.0–7.0).

This study suggests that pH does not affect methylation state, either in post-mortem brain or under experimentally induced extreme pH conditions *in vitro*. These findings should be of use to studies examining methylation state of DNA extracted from any human tissue where pH could be a factor.

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Promoter-Wide Hypermethylation of the Ribosomal RNA Gene Promoter in the Suicide Brain

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Abstract

Background: Alterations in gene expression in the suicide brain have been reported and for several genes DNA methylation as an epigenetic regulator is thought to play a role. rRNA genes, that encode ribosomal RNA, are the backbone of the protein synthesis machinery and levels of rRNA gene promoter methylation determine rRNA transcription.

Methodology/Principal Findings: We test here by sodium bisulfite mapping of the rRNA promoter and quantitative real-time PCR of rRNA expression the hypothesis that epigenetic differences in critical loci in the brain are involved in the pathophysiology of suicide. Suicide subjects in this study were selected for a history of early childhood neglect/abuse, which is associated with decreased hippocampal volume and cognitive impairments. rRNA was significantly hypermethylated throughout the promoter and 5′ regulatory region in the brain of suicide subjects, consistent with reduced rRNA expression in the hippocampus. This difference in rRNA methylation was not evident in the cerebellum and occurred in the absence of genome-wide changes in methylation, as assessed by nearest neighbor.

Conclusions/Significance: This is the first study to show aberrant regulation of the protein synthesis machinery in the suicide brain. The data implicate the epigenetic modulation of rRNA in the pathophysiology of suicide.

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Introduction

Suicide is a leading cause of death, particularly in males [1,2]. Although many suicide subjects have a diagnosable psychiatric illness, most persons with a psychiatric disorder never attempt suicide [2]. Suicidal behavior aggregates in families [1], and studies of twins show that monozygotic individuals have a greater concordance for suicide completion and suicide attempts compared to dizygotic individuals [2–4]. Non-genetic familial factors, including a history of abuse or neglect during childhood, are also risk factors for suicidal behavior [5,6]. Similarly, childhood abuse is associated with an increased risk for psychopathology [7,8] and altered neural development [9].

Several lines of evidence suggest that changes in gene expression in the brain occur in the context of psychiatric disorders and suicide [10–15]. Alterations in gene regulation can be caused by epigenetic programming of gene expression in response to environmental exposure, including social and physical adversity [16]. The genome is epigenetically programmed by changes in the chromatin state and by a pattern of modification of the DNA molecule itself through methylation [17]. DNA methylation is a stable epigenetic mark associated with long-lasting silencing of gene expression [18]. In rodents, genes responsive to differences in the quality of maternal care early in life are altered through epigenetic mechanisms [19,20]. In the human brain, aberrant DNA methylation of specific genes also occurs in the context of psychiatric disorders [21–26]. Decreased expression of ribosomal RNA (rRNA), a bottleneck gene for protein production in the cell, occurs in patients with mild cognitive impairment and early Alzheimer's disease [27,28].

DNA methylation can regulate gene expression in two ways. One is site-specific methylation, involving direct interference with the binding of transcription factors [29]. The second is site-independent promoter-wide methylation, attracting methylated DNA binding proteins and leading to an inactive chromatin structure. In the latter case, the density of methylated CpGs determines the extent of gene silencing [30]. Both mechanisms can regulate rRNA expression. Previous work in cultured mouse cells indicated that rRNA is regulated by methylation of a single CpG dinucleotide at position −133 residing at the upstream control element (UCE) [31]. In human cell culture, the transcriptionally active fraction of rRNA promoters associated with RNA polymerase I (pol I) is completely unmethylated whereas the fraction not associated with pol I is almost completely methylated [32], thus determining transcription by defining the fraction of unmethylated rRNA. Mouse and human rRNA promoters show

Table 1. Demographic characteristics and psychiatric diagnoses.[1]

	Suicide		Control	
Male/Female	18/0		12/0	
Age (years)	34 ± 9		36 ± 12	
PMI (hours)	24 ± 5.3		23 ± 5.9	
pH	6.4 ± 0.4		6.5 ± 0.2	
Childhood Abuse/Neglect	18/18	100%	0/12	0%
Mood Disorder	14/18	78%	3/12	25%
Alcohol/Drug Abuse Disorder	12/18	67%	5/12	42%
Anxiety Disorder	3/18	17%	1/12	8%

The values are mean ± SD.

[1]The number of subjects in each group represents the total number of subjects used for methylation and expression analysis. Additional subjects used for expression analysis did not differ from the other subjects in any of the listed measures (see Materials and Methods for details; P's>0.05).

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different CpG densities in the core promoter and UCE (3 in the mouse and 26–28 in the human [31,33]). Thus, although in both species complete methylation of CpGs in the promoters characterizes inactive alleles, the number of CpGs involved is different suggesting a different mode of regulation by DNA methylation.

In the present study, we tested the hypothesis that rRNA in the human hippocampus of suicide subjects with a history of childhood abuse or severe neglect and controls who died suddenly of unrelated causes without a history of childhood abuse or severe neglect is differentially methylated and expressed. Within the genome there are over 400 copies of the rRNA gene, encoding a large pre-rRNA transcriptional unit (45S) whose expression is tightly regulated by methylation [31–34]. We particularly examined the core promoter region and UCE of rRNA because it is involved in the regulation of all pol I transcribed copies of rRNA by methylation [33]. Our strategy was to sample the average methylation pattern of the rRNA promoter at single nucleotide resolution to determine CpG site specificity in the regulation of rRNA gene expression in the brains of suicide subjects and controls. The results implicate the epigenetic modulation of rRNA in the pathophysiology of suicide.

Results

The subject characteristics are presented in **Table 1**. There were no significant differences in post-mortem interval (PMI), brain pH, or age between suicide subjects and controls (P's>0.05).

Genotyping of the rRNA promoter

Because alterations in rRNA function may occur due to both genetic and epigenetic differences, the rRNA promoter region from each suicide subject and control was sequenced. No sequence variants were seen among subjects (**Fig. 1**; also see **Fig. S1**). When the sequence was compared to the published reference sequence for the rRNA promoter region (Genebank accession number: U13369) a few discrepancies were discovered. Notably, two CpG dinucleotides were not found in the sequenced DNA (**Fig. 1**). One CpG dinucleotide between the CpG dinucleotides in positions −108 and −103 was simply absent, and a second just upstream of the CpG dinucleotide in position 23 was found to be a C/T nucleotide substitution. Thus, of the 28 CpG dinucleotides in the published sequence, 26 CpG dinucleotides were present in the samples. As all subjects in our study were of French-Canadian origin, a population with a well established founder effect [35], it is

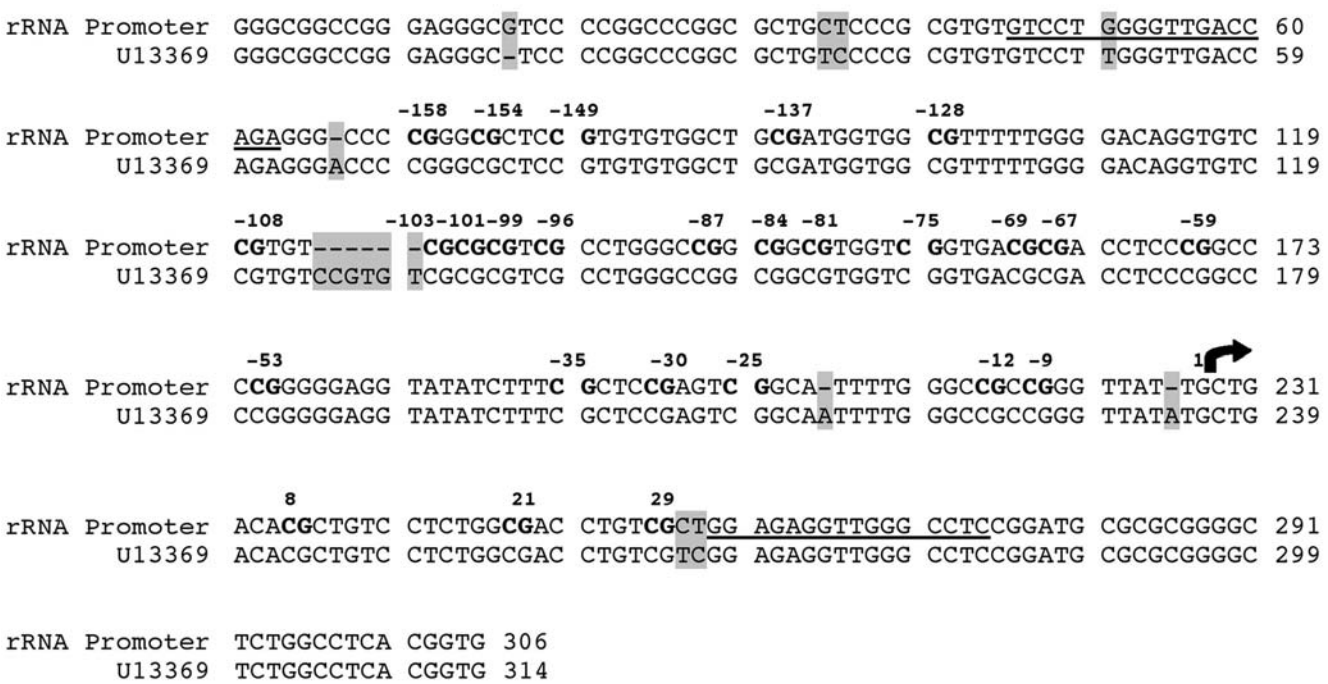

Figure 1. Genotyping of the rRNA promoter. The rRNA promoter sequence was identical for all suicide subjects and controls. The sequence derived from genotyping is shown above the published rRNA sequence, indicating consensus sequences for primers used for sodium bisulfite mapping (underline) and CpG dinucleotides (bold font), with locations marked relative to the transcription start site (arrow). Differences with the published rRNA sequence, U13369, are highlighted in grey, and the base pair length of each sequence is listed on the right side.

doi:10.1371/journal.pone.0002085.g001

likely that these differences reflect population-specific variants with regards to the reference sequence. For each subject, sequences overlapping the region targeted by primers after bisulfite conversion were identical to the published sequence, except for the presence of a G/T conversion in the forward primer that was present in all subjects, thus eliminating potential primer bias between subjects in sodium bisulfite mapping.

The rRNA Promoter is Hypermethylated in the Hippocampus of Suicide Subjects

DNA methylation can affect expression via specific methylation of distinct CpG sites [29] and/or via regional site-independent changes in the overall density of methylation [30]. Previous work in cell culture on the state of human rRNA promoter methylation showed regional differences in methylation between active and inactive rRNA promoters [32]. To determine whether the pattern of methylation of single nucleotides and/or the overall methylation of rRNA differed between suicide subjects and controls, the rRNA promoter was examined at single nucleotide resolution by sodium bisulfite mapping (**Fig. 2**). The rRNA promoter was heavily methylated throughout the promoter and 5′ regulatory region in the hippocampus of suicide subjects in comparison with that of controls ($F(1) = 191.04$, $P<0.0001$, **Fig. 2, 3A**). Twenty-one out of 26 CpG sites were significantly more methylated in suicide subjects compared to controls, whereas no CpG was more methylated in controls relative to suicide subjects ($F(25) = 11.01$, $P<0.001$; **Fig. 3A**). An analysis of the effect size for each CpG site revealed that these 21 sites did not differ in the magnitude of the methylation difference between groups at each CpG site (P's>0.05).

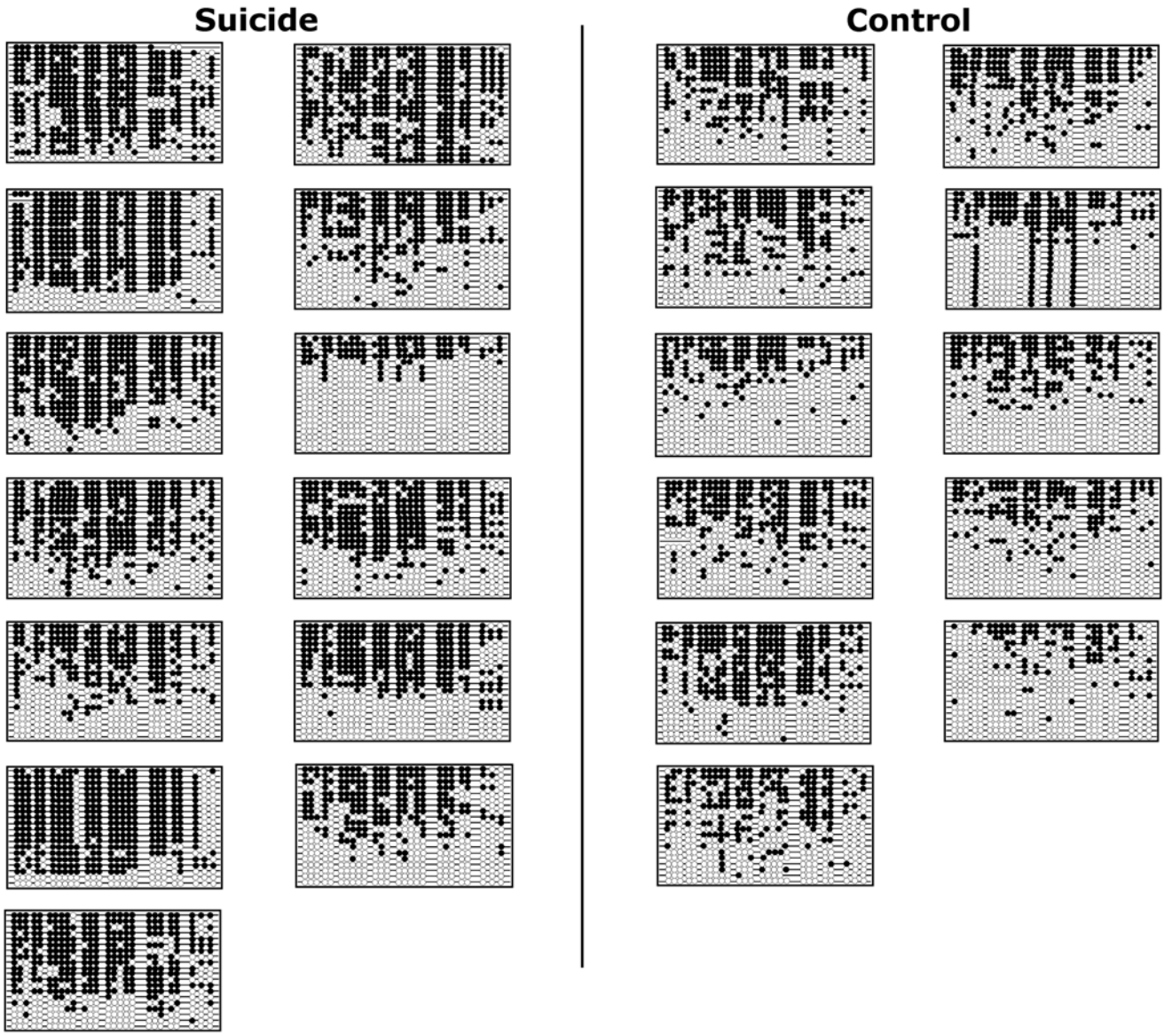

Figure 2. Sodium bisulfite mapping of the rRNA promoter in suicide subjects and controls. Twenty clones were sequenced for each suicide subject (left side) and control (right side), from 2 to 3 independent PCR reactions. Each line represents one clone. Circles representing CpG dinucleotides follow the 5′ to 3′ order of the rRNA promoter sequence for methylated CpG dinucleotides (filled circles), and unmethylated CpG dinucleotides (open circles).
doi:10.1371/journal.pone.0002085.g002

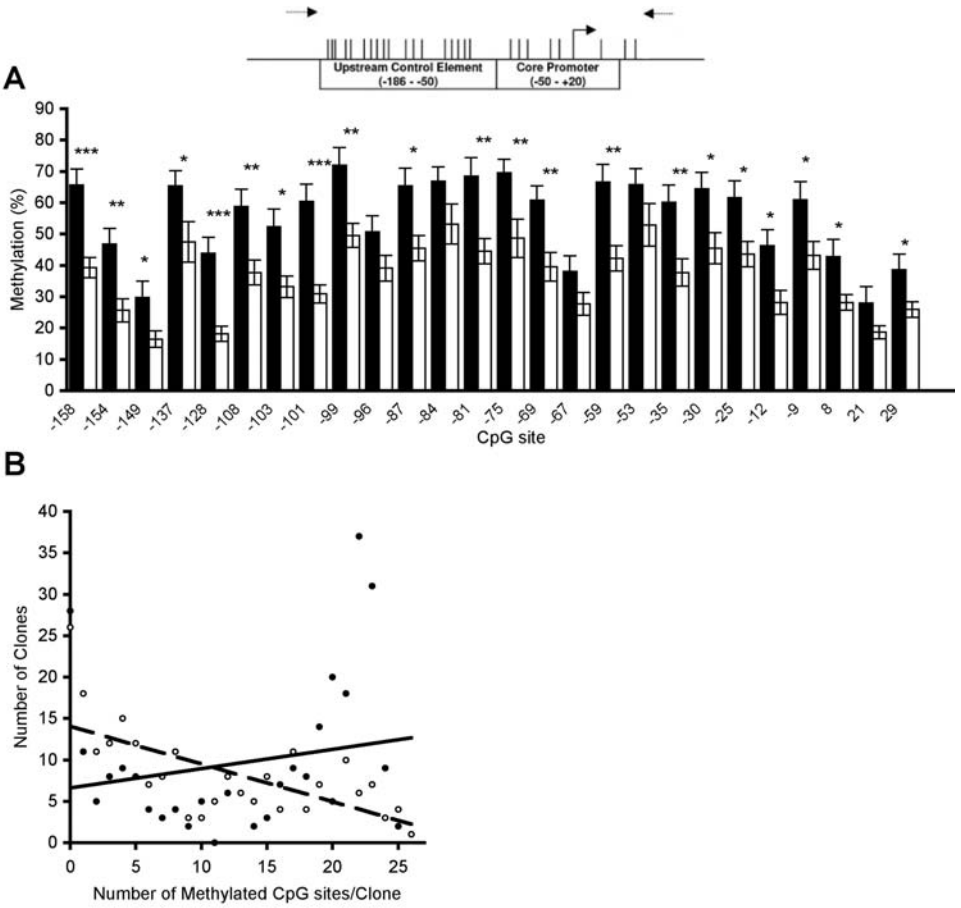

Figure 3. Hypermethylation of the rRNA promoter in suicide subjects relative to controls. (**A**) (above) Vertical lines indicate locations of CpG dinucleotides on the rRNA promoter relative to the transcription start site, indicated with the solid arrow, with primer pairs used for bisulfite mapping marked by dashed arrows. (below) Average percentage of methylation for each CpG site, for suicide subjects (N = 13; black bars) and controls (N = 11; white bars). Data are expressed as mean ± S.E.M. *, P<0.05; **, P<0.01; ***, P<0.001, measured by ANOVA. (**B**) Multiple regression analysis of the number of methylated CpGs per clone and the number of clones shows a significant interaction between suicide subjects (20 clones × 13 subjects, N = 260 total clones; filled circles) and controls (20 clones × 11 subjects, N = 220 total clones; open circles). There are 26 circles per group, as clones are grouped according to methylation status.
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A greater number of sequenced clones were hypermethylated in the suicide subjects, whereas control subjects showed a greater number of hypomethylated clones (**Fig. 3B**). An analysis of the regression slopes between groups revealed a significant ($F(1) = 7.33$, $P<0.01$) interaction between the number of methylated CpG sites per clone for suicide subjects compared to controls. These data indicate a dramatic increase in the ratio of methylated to unmethylated clones among suicide subjects across most CpG sites.

To determine whether the state of methylation of specific CpG methylation sites differed across the rRNA promoter between suicide subjects and controls or whether all CpG sites differed similarly between the two groups, the relationship between groups of the average percentage of methylation for each CpG site was investigated. There was a strong linear relationship between the means of the two groups ($R = 0.92$, $P<0.00001$; **Fig. 4**) demonstrating a similar difference in the state of methylation of all CpG sites in between the groups. No single CpG site stood out as being particularly different between the groups suggesting no site selectivity of methylation in the rRNA of suicide subjects. Instead, the overall level of methylation throughout the promoter and regulatory region differed between the groups. These data showing a lack of site-specificity are consistent with our recent analysis of the state of methylation of rRNA genes in cultured cells [32].

Specificity of Hippocampal rRNA methylation by Analysis of Cerebellum rRNA and Genome-Wide Methylation

To examine the anatomical specificity of the differences in rRNA methylation between suicides and controls in the hippocampus, rRNA promoter methylation was examined in the cerebellum, a region not primarily associated with psychopathology. Individuals from the suicide subjects group who showed large differences in hippocampal rRNA promoter methylation by contrast to those in the control group ($t(6) = 4.12$, $P<0.01$; **Fig. 5A**) were selected to test whether these differences would be conserved in another brain region. In contrast to the hippocampus, there was no significant difference in the percentage of methylated CpG sites between suicide subjects and controls in the cerebellum ($t(6) = 0.55$, $P = 0.6$; **Fig. 5B**). There was no significant correlation between levels of methylation in the hippocampus and those in the cerebellum ($R = 0.11$, $P = 0.78$), showing anatomical specificity in the hypermethylation of the rRNA promoter in hippocampus of suicide subjects. The interaction between the regression slopes for the number of methylated CpG sites per clone was not significant ($F(1) = 0.26$, $P = 0.61$; **Fig. 5C**), indicating that there was no difference in the ratio of unmethylated to methylated clones between groups in the cerebellum.

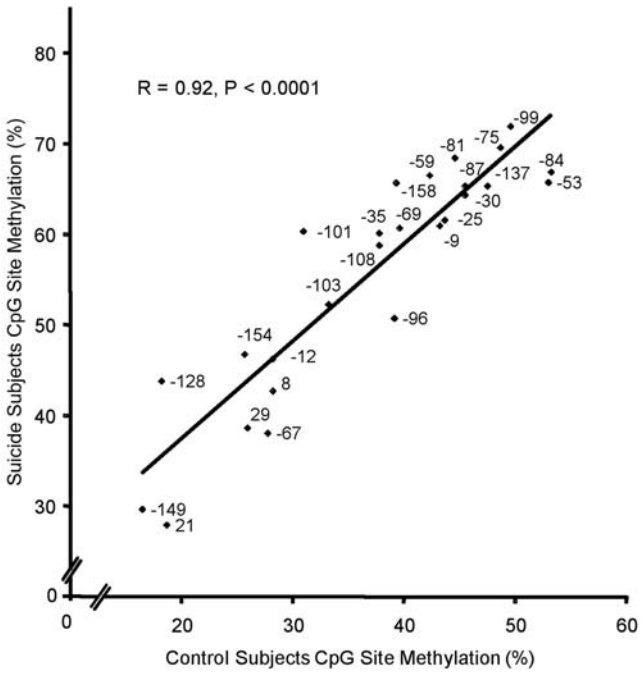

Figure 4. Site-independent hypermethylation of the rRNA promoter in suicide subjects. Positive correlation between suicide and control rRNA promoter methylation percentage across CpG sites (N = 26), showing a conserved hypermethylation in suicide subjects throughout the promoter region. Each data point is labeled with the position of each CpG dinucleotide in the rRNA promoter, relative to the transcription start site.
doi:10.1371/journal.pone.0002085.g004

To determine whether the observed differences in methylation of the rRNA promoter in the hippocampus reflect genome-wide differences in methylation between suicide subjects and controls, nearest neighbor analysis of the overall percentage of methylated cytosines was performed for each subject. Nearest neighbor analysis revealed no significant difference between suicide subjects and controls in the overall percentage of methylated cytosines ($t(22) = 0.54$, $P = 0.59$), and there was no significant correlation between the percentage of rRNA promoter methylation and genome-wide methylation ($R = 0.07$, $P = 0.78$), revealing specificity in the regulation of the rRNA promoter by methylation in the suicide brain (**Fig. 5D**).

rRNA Methylation Does Not Vary With Psychiatric Diagnoses

Next, the relationship between methylation and psychiatric diagnoses was examined (**Table 1**). Mood disorders and substance abuse disorders are risk factors for suicide and have also been linked to alterations of DNA methylation in several genes [24,25,36,37]. There were no significant differences within the suicide subjects or the controls when the overall percentages of rRNA methylation of those with mood disorders were compared to those without mood disorders as well as among those with substance abuse disorders compared to those without substance abuse disorders (all P's>0.05).

rRNA Expression is Downregulated in Suicide

Because it has been established in cell culture that methylation of the rRNA promoter regulates the transcription of rRNA [31–34], rRNA expression in suicide subjects and controls was investigated. rRNA expression was significantly higher in controls than in suicide subjects ($t(23) = 2.16$, $P<0.05$; **Fig. 6A**). Correlational analysis revealed a trend for a relationship between the overall percentage of methylation and rRNA expression ($R = -0.21$, $P>0.05$; **Fig. 6B**), indicating that rRNA expression may be regulated by methylation and additional epigenetic factors. There was no relationship between and PMI, brain pH, or age and rRNA expression (P's>0.05).

Discussion

The data reveal evidence for DNA hypermethylation of the rRNA promoter region in the hippocampus of suicide subjects with histories of childhood abuse or severe neglect relative to controls (victims of sudden, accidental death with no history of abuse or neglect). Although our findings are largely based on correlational studies indicating an association between psychopathology and methylation, these data are consistent with growing evidence suggesting that alterations in cytosine methylation mediate biological processes associated with psychopathology [38].

Since DNA methylation is a highly stable mark, the bond between a methyl group and cytosine ring being one of the most stable chemical bonds [18], the differences in methylation are unlikely to be a consequence of conditions immediately preceding death or during the postmortem interval. No reaction which could spontaneously demethylate 5-methylcytosine in DNA has ever been described. Our data indicate that post mortem pH does not affect DNA methylation (CE POM VD MJM MS and GT, unpublished observations). Indeed, post-mortem interval, brain pH, or age did not differ between suicide subjects and controls.

The increase in hippocampal rRNA promoter methylation among suicide subjects appears to occur in the absence of obvious site-specific effects on particular CpG sites. The results are consistent with data in cell culture showing that transcriptionally active rRNA promoters are completely unmethylated while transcriptionally inactive molecules are completely methylated in a promoter-wide manner without any obvious site selectivity [32]. In contrast to the situation in humans reported here and previously reported in human cells in culture, the situation in mouse is different. In the mouse, a site-specific change in methylation is sufficient to mediate silencing of the rRNA promoter [31].

Importantly, the changes in rRNA promoter methylation do not reflect a genome-wide change in methylation, as nearest neighbor analysis revealed no differences in overall levels of methylation in suicide subjects relative to controls. Moreover, this difference in the methylation of the rRNA promoter shows anatomical specificity. When the rRNA methylation status for subjects with large methylation differences in hippocampus was assessed in the cerebellum, suicide subjects and controls showed similar levels of rRNA methylation. In contrast to the hippocampus, the number of methylated CpG sites observed per clone was similar between suicide subjects and controls in the cerebellum. As a part of the brain not primarily associated with neuroplastic changes influencing psychopathology, this result indicates that rRNA methylation differences between the groups are specific to the hippocampus.

In addition to the difference in methylation, suicide subjects showed impaired hippocampal rRNA expression compared to controls. The decrease in gene expression was associated with increased methylation of the rRNA promoter sequence, as indicated by a trend for a linear correlation between the overall percentage of methylation and gene expression. Although a trend was evident, the results do not exclude other known epigenetic mechanisms influencing rRNA gene expression. For example, in

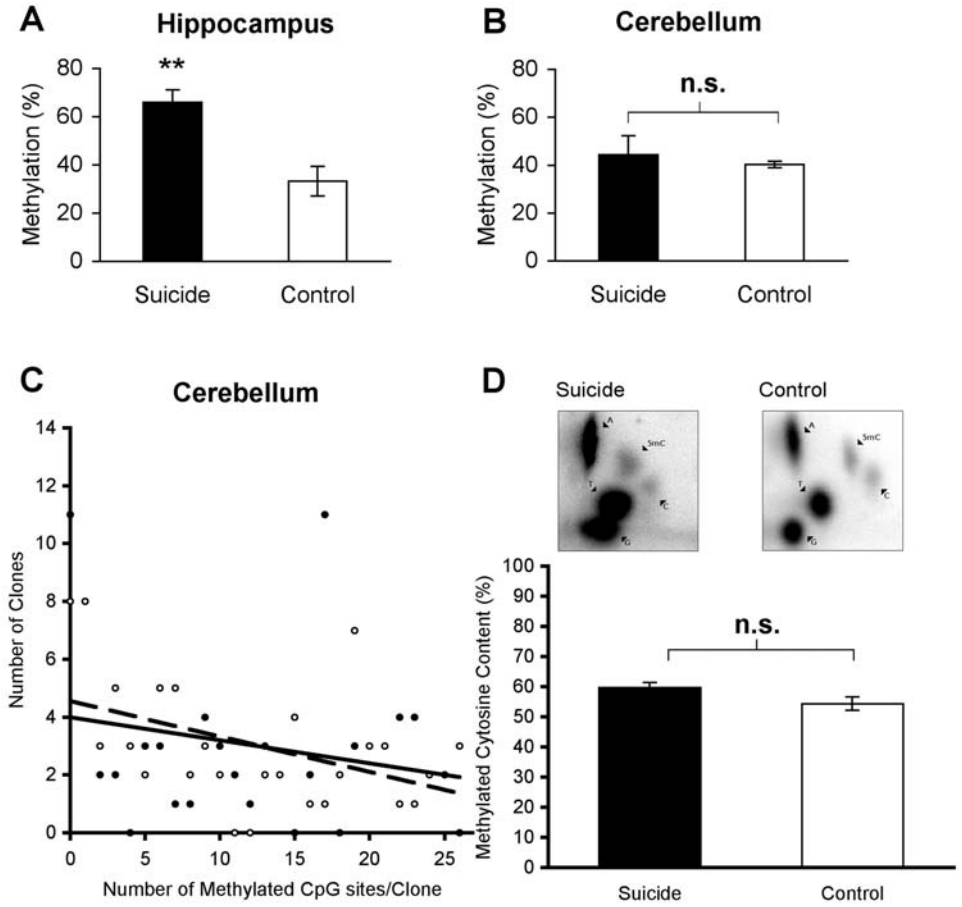

Figure 5. Anatomical and Genomic specificity of rRNA hypermethylation. Average percentage of rRNA promoter methylation for selected subjects with large methylation differences in the hippocampus (**A**) and in the cerebellum (**B**) of suicide subjects (N = 4, black bars) and controls (N = 4, white bars) for the same subjects. Data are expressed as mean ± S.E.M. **, $P<0.01$, measured by unpaired t-test. (**C**) Multiple regression analysis shows a similar negative relationship between the number of methylated CpGs per clone and the number of clones in cerebellum samples from suicide subjects (20 clones × 4 subjects, N = 80 total clones; filled circles) and controls (20 clones × 4 subjects, N = 80 total clones; open circles). There are 26 circles per group, as clones are grouped according to methylation status. (**D**) (above) Representative images of genome-wide methylation in the hippocampus for a suicide subject and a control, showing cytosine (C) and 5-methylcytosine (5 mC) content used for nearest neighbor analysis. (below) Quantification of the percentage of methylcytosine, following the formula: [(5-methylcytosine) × 100]/(5-methylcytosine + cytosine), shows no difference between suicide subjects (N = 13, black bar) and controls (N = 11, white bar) in genome-wide levels of methylation ($P>0.05$), measured by unpaired t-test.
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cultured cells pharmacological manipulation of the acetylation status of histone 4 influences rRNA expression [31,39].

In this study, we selected suicide subjects with a history of early childhood neglect/abuse. Childhood abuse in humans is associated with decreased hippocampal volume, as well as with cognitive impairments [9]. This influence of childhood adversity and epigenetic aberrations later in life supports the hypothesis that, similar to what was observed in rodents [19,20,40], early childhood events in humans alter epigenetic markings in the brain. It is tempting to speculate that epigenetic processes mediate effects of social adversity during childhood on the brain that persist into adulthood and are known to enhance suicide risk [41,42].

Epigenetic differences might be driven by genetic differences as well as by other environmental and dietary factors. All suicide subjects in our study displayed the same genomic sequence in the rRNA promoter region examined. Additional genetic polymorphisms in other regions of the rRNA gene, including among individual rRNA gene clusters, may play a role in rRNA function. The structure and length of rRNA gene clusters varies between individuals [43,44], however, the relationship between rRNA promoter methylation or rRNA expression and this additional level of organizational complexity in rRNA is less clear. Genetic abnormalities in rRNA gene cluster organization are associated with increased rRNA methylation during cellular senescence [45]. However, the lack of difference between groups in the cerebellum argues against such genetic differences among these individuals. Another factor that may influence the methylation status of individuals is medication prescribed in the treatment of psychiatric disorders. The mood stabilizing effect of sodium valproate, a potent histone deacetylase (HDAC) inhibitor known to indirectly influence DNA methylation [46,47] via chromatin modification and used in the treatment of bipolar disorder, and the noted antidepressive effect of S-adenosyl methionine, a methyl donor in the DNA methylation reaction and an inhibitor of active demethylation [48] , suggest a role for DNA methylation in mood regulation [49]. Although none of the subjects in the present study had been treated with these pharmacological agents, the possibility of epigenetic regulation by other pharmacological interventions should not be discounted. Our data do not exclude these alternative hypotheses.

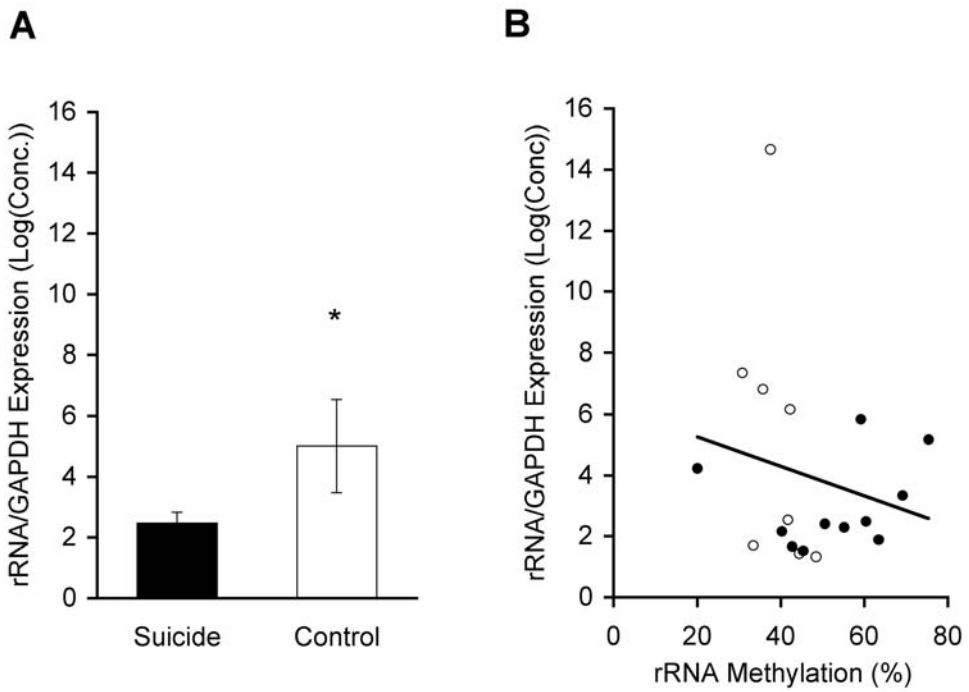

Figure 6. rRNA expression is downregulated in suicide. (**A**) Suicide subjects (N = 16, black bar) showed significantly less rRNA expression than controls (N = 9, white bar); Data expressed as mean ± S.E.M. *, $P<0.05$, measured by unpaired t-test. (**B**) Among subjects where both methylation and expression of rRNA were analyzed, the correlation between rRNA promoter methylation percentage and expression showed a trend for an inverse relationship between methylation percentage and expression of rRNA in suicide subjects (N = 11, filled circles) and controls (N = 8, open circles).
doi:10.1371/journal.pone.0002085.g006

In summary, these data reveal increased promoter-wide methylation of the rRNA promoter as well as decreased rRNA gene expression in suicide subjects. The results of the psychological autopsy suggest a developmental origin, however, at time this is speculation based on samples that differ along a wide range of experiential and potentially genetic dimensions. To date, our data are merely consistent with the hypothesis that early life events can alter the epigenetic status of genes that mediate neural functions, and thus contribute to individual differences in the risk for suicide.

Materials and Methods

Subjects and Sample Preparation

Hippocampal samples obtained from the Quebec Suicide Brain Bank included: 13 suicide subjects and 11 controls matched for post-mortem interval (PMI), gender, and age (**Table 1**). Cerebellar samples were also obtained for 4 of the same suicide subjects and 4 of the same controls as those used for hippocampal analysis. An additional 6 hippocampal samples consisting of 5 suicide subjects and 1 control were also obtained from the Quebec Suicide Brain Bank for RNA expression analysis, to compensate for the removal of 5 subjects (2 suicide and 3 controls) due to low RNA quality. All samples were from male suicide and control subjects of French-Canadian origin that were processed as previously described [50]. Samples were dissected at 4°C and stored in plastic vials at −80°C until analysis. All samples were processed and analyzed blind to demographic and diagnostic variables. Possible confounders that were examined included PMI, brain pH, and age at death of the donor (**Table 1**). This study was approved by our local Institutional Review Board and signed informed consent was obtained from next of kin.

To be included in this study, all subjects had to die suddenly, with no medical or paramedic intervention. Suicide as the cause of death was determined by the Quebec Coroner's Office. Psychiatric diagnoses were obtained by means of the SCID I [51] and SCID II [52] interviews adapted for psychological autopsies, which is a validated method to reconstruct psychiatric history by means of extensive proxy-based interviews, as outlined elsewhere [41]. In addition, to be considered in this study, all suicide subjects and none of the controls had to have a positive history of childhood abuse or severe neglect, as determined by structured interviews using the Childhood Experience of Care and Abuse (CECA) [53] questionnaire adapted for psychological autopsies, as described elsewhere [54].

Genotyping of the rRNA promoter region

Genomic DNA was extracted (DNeasy, Qiagen) according to the manufacturer's protocol. Primers for PCR were directed against the rRNA gene promoter (Genebank accession number U13369) using the following sequences: 5′-GTG TGT CCC GGT CGT AGG-3′; antisense: 5′-GTC ACC GTG AGG CCA GAG-3′. Primers were selected on the basis that they covered a 400bp region that included the region selected for sodium bisulfite analysis, including the regions covered by sodium bisulfite primers. The resulting PCR products for each subject were sequenced bidirectionally using the forward and the reverse primer on an ABI 3100 genetic analyzer (Applied Biosystems) and following the manufacturer's instructions. Genetic variation was assessed throughout the rRNA promoter region used for bisulfite analysis by alignment of genomic DNA with the previously published rRNA gene promoter sequence using freely available software (CLC Workbench, CLC bio).

Sodium Bisulfite Mapping of DNA Methylation Status

Genomic DNA was extracted (DNeasy, Qiagen) and sodium bisulfite conversion of genomic DNA was performed as previously described [55,56] for 13 suicide subjects and 11 controls for hippocampal samples and for 4 suicide subjects and 4 controls for cerebellum samples. Primers for PCR were directed against the rRNA gene promoter using the following sequences: sense: 5′-GTT TTT GGG TTG ATT AGA-3′; antisense: 5′-AAA ACC CAA CCT CTC C-3′ [32]. Because the primers did not contain CpG dinucleotides, methylated and unmethylated sequences amplified with equal efficiency. The resulting product was excised, purified, subcloned, and transformed (TA cloning kit, Invitrogen). Individual clones were extracted and sequenced (CEQ 8800, Beckman-Coulter) according to the manufacturer's protocol. Twenty clones were sequenced for each subject from 2 to 3 independent PCR reactions. To ensure that the bisulfite conversion was complete, only clones in which all cytosine residues in non-CpG dinucleotides had been converted to thymidine were included in the analysis.

Nearest Neighbor Quantification of Methylated Cytosine Content

Genome-wide levels of 5-methylcytosine were quantified as previously described [57]. Briefly, genomic DNA from the same subjects as those used for bisulfite analysis was subjected to MBoI restriction enzyme digestion (recognition sequence: NGATCN), incubated with a ^{32}P-labelled oligonucleotide, loaded onto TLC phosphocellulose plates, and separated by chromatography. Reactions were repeated in triplicate. The intensities of 5-methylcytosine and cytosine spot densities were analyzed using a PhosphoImager screen followed by Image Quant image analysis. For each subject, levels of methylated cytosine were tabulated as a percentage of total cytosine content, following the formula: [(5-methylcytosine) × 100]/(5-methylcytosine + cytosine).

RNA Extraction and Quantitative Reverse Transcription PCR (qRTPCR) of rRNA Gene Expression

RNA extraction was performed using Trizol (Invitrogen) followed by Dnase I treatment, and cDNA conversion was performed using random hexamer primers (Invitrogen) according to the manufacturer's instructions (Roche Molecular Biochemicals). The subjects were the same as those used for the bisulfite mapping study, however, RNA samples from subjects with RNA Integrity Numbers (RINs) lower than 5.0 or brain pH less than 6.0 were excluded from analysis (N = 2 suicide subjects and N = 3 control subjects), consistent with previously described criteria for exclusion [50]. Because of the low number of remaining subjects and preliminary data indicating a trend for lower rRNA expression in the suicide subjects used for bisulfite analysis, we included an additional 5 suicide subjects (total N = 16) and 1 control subject (total N = 9) for rRNA expression analysis. Primers were directed against the rRNA gene using the following sequences: sense; 5′-TTC TCT AGC GAT CTG AGA GGC GT-3′, antisense; 5′-TAC CAT AAC GGA GGC AGA GAC AGA-3′ and GAPDH (Genebank accession number NM_002046) using the following sequences: sense; 5′-GAA GGT GAA GGT CGG ACT C-3′, antisense; 5′-GAA GAT GGT GAT GGG ATT TC-3′. A master mix, containing the cDNA, 10 mM Tris-Cl, 50 mM KCl, 2.0 mM MgCl2, 0.2 mM dNTPs, 5X SYBR Green I Solution, HotStart Taq DNA polymerase (Superarray Bioscience Corporation), and 0.2 μM of the sense and antisense primers, were loaded into LightCycler capillaries (Roche Molecular Biochemicals). For the rRNA gene, the qRTPCR protocol (LightCycler Software 3.5, Roche Molecular Biochemicals) consisted of initial HotStart Taq DNA polymerase activation cycle (15 min, 95°C, with a temperature transition rate of 20°C/sec) followed by 36 cycles of denaturation (30 sec, 95°C, with a temperature transition rate set at 20°C/sec), annealing (30 sec, 55°C, with a temperature transition rate set at 20°C/sec) and elongation (30 sec, 72°C, with a temperature transition rate set at 20°C/sec). A single fluorescence reading was acquired at the end of each elongation step. Subsequently, the PCR products were melted using the following program: 95°C with a temperature transition rate of 20°C/sec, 65°C, with a temperature transition rate of 20°C/sec followed by 95°C, with a temperature transition rate of 0.1°C/sec. The presence of a single melting peak followed by analysis on 1.5% agarose gel confirmed product specificity. For the GAPDH gene, the above procedure was identical except for annealing temperatures of 51°C and 61°C during the PCR and melting steps, respectively. Reactions were repeated in triplicate. Reactions were also carried out in the absence of reverse transcriptase to verify the absence of genomic DNA contamination. To determine the relative concentrations of rRNA gene expression, a standard curve of 10-fold serial dilutions of a mixture of each of the sample cDNA was used to plot the relative Ct value for each gene on the y-axis and the amount of cDNA used on the x-axis. To calculate the fold-change, the relative amount of rRNA product was divided by the relative amount of GAPDH for each subject.

Statistical Analyses

Statistical analyses were carried out using Statview (Cary, NC). For DNA methylation analysis, a factorial ANOVA was carried out with the percentage of methylation as the dependent variable and group (suicide subjects and controls) as the between groups factor. The data were then subjected to Bonferroni Post-hoc analysis to examine methylation status between groups across all CpG sites. A standardized effect size and associated 95% confidence interval of the methylation differences between suicide subjects and controls was calculated for each CpG site using the difference between group means divided by a pooled standard deviation corrected for bias, according to previously described methods [58,59]. The analysis of the relationship between DNA methylation at each CpG site between suicide subjects and controls was conducted using linear regression, as were analyses of the relationships between DNA methylation, expression, PMI, brain pH, and age. To identify possible diagnostic variables influencing methylation status, factorial ANOVA followed by Bonferroni Post-hoc comparisons were used to compare groups of subjects with different clinical diagnoses. For nearest neighbor as well as rRNA expression analysis, unpaired t-tests were used to examine differences between the suicide and control groups. Data from these statistical analyses are presented as mean ± SEM. Statistical significance was determined at $P \leq 0.05$.

Supporting Information

Figure S1 The published rRNA promoter sequence U13369 is followed by the sequencing results for each subject for the region examined by sodium bisulfite mapping. The base pair length of each sequence is listed on the right side and the consensus sequence at the bottom of the sequencing results.
Found at: doi:10.1371/journal.pone.0002085.s001 (0.38 MB PDF)

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Author Contributions

Conceived and designed the experiments: MM MS GT. Performed the experiments: PM AS AU TH. Analyzed the data: PM AS MS. Contributed reagents/materials/analysis tools: MS CE GT. Wrote the paper: MM MS PM AS GT.

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BRAIN RESEARCH

Review

Diet and the epigenetic (re)programming of phenotypic differences in behavior

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ABSTRACT

Phenotypic diversity is shaped by both genetic and epigenetic mechanisms that program tissue specific patterns of gene expression. Cells, including neurons, undergo massive epigenetic reprogramming during development through modifications to chromatin structure, and by covalent modifications of the DNA through methylation. There is evidence that these changes are sensitive to environmental influences such as maternal behavior and diet, leading to sustained differences in phenotype. For example, natural variations in maternal behavior in the rat that influence stress reactivity in offspring induce long-term changes in gene expression, including in the glucocorticoid receptor, that are associated with altered histone acetylation, DNA methylation, and NGFI-A transcription factor binding. These effects can be reversed by early postnatal cross-fostering, and by pharmacological manipulations in adulthood, including Trichostatin A (TSA) and L-methionine administration, that influence the epigenetic status of critical loci in the brain. Because levels of methionine are influenced by diet, these effects suggest that diet could contribute significantly to this behavioral plasticity. Recent data suggest that similar mechanisms could influence human behavior and mental health. Epidemiological data suggest indeed that dietary changes in methyl contents could affect DNA methylation and gene expression programming. Nutritional restriction during gestation could affect epigenetic programming in the brain. These findings provide evidence for a stable yet dynamic epigenome capable of regulating phenotypic plasticity through epigenetic programming.

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Abbreviations: HAT, Histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; DNMT, DNA methyltransferase; SAM, S-adenosylmethionine; HDACi, HDAC inhibitor; CBP, CREB binding protein; TSA, Trichostatin A; MBD2, METHYLATED DOMAIN DNA BINDING PROTEIN 2; NGFI-A, NERVE GROWTH FACTOR-INDUCIBLE PROTEIN A; LG, Licking/Grooming

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Contents

1. Genes, gene expression programs, diet and mental health

Different cell types execute distinct patterns of gene expression that are highly responsive to developmental, physiological, pathological and environmental cues. The combination of mechanisms that confers long-term programming to genes leading to a change in gene function without a change in gene sequence is termed here epigenetic. The epigenetic programming of gene expression is somewhat dynamic in response to environmental exposures — especially though perhaps not exclusively during fetal development and early in life. Thus, much of the phenotypic variation seen in human populations might be caused by differences in long-term programming of gene function rather than the genetic sequence *per se*. Any analysis of inter-individual phenotypic diversity should take into account epigenetic variations in addition to genetic sequence polymorphisms (Meaney and Szyf, 2005b).

Some critical environmental exposures such as variations in maternal behavior and diet could alter the progression of epigenetic programming during development postnatally as well as in utero. Thus, variation in environmental exposures during these critical periods could result in epigenetic and therefore phenotypic differences later in life. It stands to reason that exposure to nutritional deprivation would affect the epigenetic machinery during development. Recent data suggest that psychosocial exposures early in life also impact the epigenome resulting in differences in epigenetic program and as a consequence in behavioral differences later in life (Meaney and Szyf, 2005a). Thus, certain behavioral pathologies might be a consequence of early in life exposures that alter epigenetic programming.

It is important to understand the mechanisms driving variations in epigenetic programming in order to identify the behavioral pathologies that result from such mechanisms. Unlike genetic mechanisms, epigenetic mechanisms are dynamic and thus potentially reversible and amenable to therapeutic intervention (Szyf, 2001). Because various drugs used in the treatment of psychiatric disorders such as schizophrenia and mood disorders have known epigenetic effects, interventions targeting the epigenetic machinery could have important consequences for normal cognitive function. Thus, components of diet that influence the epigenetic machinery should be considered interventions that could affect mental as well as physical health. Once the rules governing the effects of environmental exposures on epigenetic processes are understood, it might be possible to design behavioral and nutritional strategies to prevent and reverse deleterious environmentally driven epigenetic alterations.

2. The epigenome

The epigenome consists of chromatin, a protein-based structure around which wrapped the DNA, and its modifications as well as a covalent modification of cytosines residing at the dinucleotide sequence CG in DNA itself by methylation (Razin, 1998). These modifications determine the accessibility of the transcriptional machinery to the genome. Recently, an additional level of epigenetic regulation by small non-coding RNAs termed microRNA has been discovered (Bergmann and Lane, 2003). microRNA expression is itself regulated by epigenetic factors such as DNA methylation and chromatin structure (Saito and Jones, 2006). Therefore microRNAs should be considered under the headings of chromatin and DNA methylation, as they also act by changing chromatin structure (Chuang and Jones, 2007).

2.1. Chromatin structure and the histone code

The basic building block of chromatin is the nucleosome, which is formed of an octamer of histone proteins. The octamer structure of the nucleosome is composed of a H3–H4 histone protein tetramer flanked on either side with a H2A–H2B histone protein dimer (Finch et al., 1977). The N-terminal tails of these histones are extensively modified by methylation (Jenuwein, 2001), phosphorylation, acetylation (Wade et al., 1997), sumoylation (Shiio and Eisenman, 2003) and ubiquitination (Shilatifard, 2006). The specific pattern of histone modifications was proposed to form a 'histone code', that delineates the parts of the genome to be expressed at a given point in time in a given cell type (Jenuwein and Allis, 2001). Similar to a genetic mutation, a change in the state of modification of histone tails around a regulatory region of a gene can silence an active gene, resulting in "loss of function", or activate a silent gene, leading to "gain of function". In addition, such modifications can also enhance or impair levels of gene expression in the absence of complete gene silencing or activation.

2.2. Chromatin remodeling and targeting

Chromatin remodeling complexes can alter the position of nucleosomes around the transcription initiation site and define its accessibility to the transcription machinery (Varga-Weisz and Becker, 2006). It is becoming clear that both gene activating complexes and gene repression complexes contain chromatin remodeling activities (Xue et al., 1998). Remodeling might be required for facilitating the interaction between histone tails and chromatin-modifying enzymes.

The state of modification at specific loci is defined through recruitment of chromatin-modifying enzymes by sequence-specific factors to specific loci. Histone modifications are catalyzed by histone-modifying enzymes such as histone acetyltransferases (HAT), which acetylate histone tails and histone deacetylases (HDAC) that deacetylate histone tails (Kuo and Allis, 1998). Another group of important enzymes are the histone methyltransferases (HMT) and the histone demethylases (Shi et al., 2004; Tsukada et al., 2006). The balance of these activities determines the state of histone modification and thus the level of expression of the associated genes.

An important point that is emerging from current studies is that the state of modification of chromatin is not dependent exclusively on the overall levels of the histone-modifying enzymes but also on the targeting of these enzymes to specific genes. Specific transcription factors and transcription repressors recruit histone-modifying enzymes to specific genes and thus define the gene-specific profile of histone modification (Jenuwein and Allis, 2001). Some environmentally regulated alterations of histone acetylation in specific promoter sequences following seizures (Huang et al., 2002; Tsankova et al., 2004) or learning (Guan et al., 2002) are likely to be caused by neurotransmitter activation of multiple signalling pathways (Crosio et al., 2003). However, such histone modifications are transient and cannot directly explain enduring early environmental programming effects. A more likely, highly stable candidate could involve modification of the genome itself.

2.3. DNA methylation and consequences for transcription

DNA methylation is part of the covalent structure of the DNA (Razin and Riggs, 1980). This differentiates it from chromatin, which is associated with DNA but is not part of the DNA molecule itself. DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) onto the 5′ position of the cytosine ring residing in most cases at the dinucleotide sequence CG (Adams et al., 1975; Cheng et al., 1993; Ho et al., 1991; Wu and Santi, 1985). DNMT1, known as a "maintenance" methyltransferase, has a preference for a hemimethylated substrate and is involved in copying DNA methylation patterns during cellular replication (Razin and Riggs, 1980). What distinguishes DNA methylation in vertebrates is the fact that not all CGs are methylated, but there is a cell-specific pattern of distribution of methylation on CG dinucleotides (Razin and Szyf, 1984).

Several lines of new data point to a model whereby DNA methylation patterns are actively maintained by DNMTs, which are targeted to methylated sequences. First, maintenance methylation of repetitive elements was shown to require the cooperation of the so called "de novo" methyltransferases DNMT3A and DNMT3B (Liang et al., 2002). Second, DNMT1 and DNMT3B were found in same complexes (Kim et al., 2002) in somatic cells. It would be difficult to explain this co-occurrence if copying the DNA methylation pattern during replication was the only methylation activity required in somatic cells. Third, not only are DNMTs targeted to specific genes by sequence-specific factors but they are also required to reside on these sequences to maintain their methylation state (Brenner et al., 2005; Burgers et al., 2006; Di Croce et al., 2002; Fuks et al., 2001; Vire et al., 2006). The targeting of DNMTs suggests that maintenance methylation is not just automatic copying of a template pattern, but it requires the positive identification of a specific sequence.

DNA methylation in critical sites silences genes by two principal mechanisms. First, methylation in critical sites inhibits the binding of transcription factors to their recognition elements (Comb and Goodman, 1990; Inamdar et al., 1991). Second, methylation of a regulatory region of DNA recruits methylated DNA binding proteins such as MeCP2 to the gene (Fujita et al., 1999; Hendrich and Bird, 1998; Nan et al., 1997; Ng et al., 1999) and chromatin modification enzymes such as HDACs which in turn introduce histone modifications, resulting in the silencing of chromatin. Thus, any random or programmed event of DNA methylation in critical sites in response to an environment insult or trigger might result in a change in phenotype similar to a mutation in the same sequence. It is possible that the dynamic equilibrium is altered by either pathological or adaptive mechanisms in response to extra and intracellular signaling.

2.4. Reversibility of DNA methylation in somatic tissues

There is general agreement that during development both de novo methylation and demethylation events shape and sculpt the mature cell-specific DNA methylation pattern (Brandeis et al., 1993; Frank et al., 1990; Kafri et al., 1993; Razin and Shemer, 1995; Razin et al., 1984). There is also evidence that DNA methylation patterns are dynamic in neurons (Levenson et al.,

2006; Miller and Sweatt, 2007; Weaver et al., 2004; 2005). However, the precise mechanisms by which this occurs continue to be a subject of debate. There has been reluctance to accept the idea that an enzymatic activity removes methyl groups directly from the cytosine ring (Wolffe et al., 1999). We previously proposed that the METHYLATED DOMAIN DNA BINDING PROTEIN 2 (MBD2) bears a demethylation activity (Bhattacharya et al., 1999; Detich et al., 2002; 2003a; 2003b). However, other groups disputed this finding (Ng et al., 1999). A number of indirect mechanisms for demethylation have also been proposed which do not require direct removal of the methyl bond (Barreto et al., 2007; Jost, 1993; Zhu et al., 2000). We propose that, as with DNMT action, targeting plays an important role in demethylase action — activity that is intimately linked to chromatin structure.

2.5. *The relationship between chromatin structure and DNA methylation*

There is a well established bidirectional relationship between DNA methylation and chromatin structure (Razin and Cedar, 1977). Since it has been known for some time that chromatin configuration is dynamic and responsive to cellular signaling pathways, this relationship provides a link between the extracellular environment and the state of DNA methylation. That is, signaling pathways that activate chromatin-modifying enzymes could potentially result in altering DNA methylation patterns. There are genetic and epigenetic data linking chromatin modeling and modifying enzymes to DNA methylation (Fuks et al., 2000; 2003; Rountree et al., 2000; Vire et al., 2006). We propose that if a sequence-specific factor which targets DNMT is inactivated, DNMT is removed from the gene and the DNA methylation equilibrium is tilted toward DNA demethylation by demethylases. For example, in cancer, the histone methyltransferase EZH2 targets DNMTs to specific sequences in DNA (Di Croce et al., 2002; Vire et al., 2006). EZH2 associates with DNMTs in silencing of tumor suppressor genes (Schlesinger et al., 2007). The targeting factors are responsive to cellular signaling pathways, thus creating a conduit between cellular and extracellular signals and the epigenetic state. Thus, maintenance of the DNA methylation pattern is at least partly an active and targeted process rather than an automatic process, as the pattern of methylation is maintained by the constitutive presence of these sequence selective factors on the target genes. Some of these factors might be responsive to intracellular signaling pathways (Fig. 1). The requirement for targeting molecules which could be responsive to different signaling pathways might explain how environmental signals and oncogenic signals might affect the DNA methylation pattern in a dynamic way throughout life. It also implies that DNA methylation patterns could be therapeutically manipulated in the absence of cell division which has significance for brain targeted DNA methylation therapeutics. At the present time, this is an idea that requires further study with respect to signaling pathways in the brain.

Similar to DNA methylation, demethylation is targeted by transacting factors to specific genes (Kirillov et al., 1996), and demethylation is facilitated by histone acetylation (Cervoni et al., 2002; Cervoni and Szyf, 2001). Pharmacological acetylation using HDAC inhibitors (HDACi) such as TSA (Cervoni and Szyf, 2001) or valproic acid (Detich et al., 2003a) triggers replication-independent active demethylation of transiently transfected in vitro methylated plasmids and causes genomic demethylation (Milutinovic et al., 2007; Ou et al., 2007).

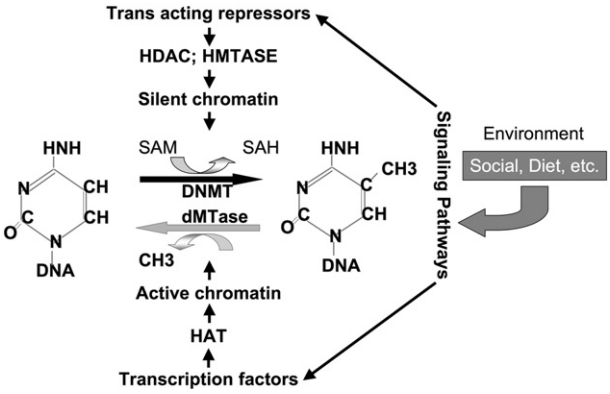

Fig. 1 – The dynamic and responsive DNA methylation pattern; a model. A balance of methylation and demethylation reactions determines the DNA methylation state. Active chromatin facilitates DNA demethylation while silent chromatin facilitates methylation. Different environmental signals trigger pathways in the cell that activate sequence-specific factors which recruit chromatin-modifying enzymes to specific loci, resulting in either activation or silencing of chromatin.

The pharmacological data with HDACi might explain why certain transcription factors target DNA demethylation to specific genes. Several transcription factors recruit HATs to genes and their mode of action is similar to TSA. By increasing histone acetylation, these factors facilitate the access of demethylation activities to their target genes, an example of which is the ubiquitous transcription factor CREB binding protein (CBP) (Braganca et al., 2003; Ogryzko et al., 1996; Purucker et al., 1990; Uchida et al., 2002; Weaver et al., 2007). As we will discuss further, such a mechanism provides a conduit through which both the chemical and the social environment could affect our epigenome and thus gene expression and function, including in neurons (Fig. 1).

2.6. *The dynamic pattern of DNA methylation in neurons*

It stands to reason that certain chemicals would interfere with DNA methylation enzymes and thus result in an alteration in DNA methylation. It is also widely accepted that chemicals as well as altered dietary intake would affect DNA methylation during gestation (Simmons, 2007) especially during gametogenesis (Anway et al., 2005) at a point when methylation machineries are highly active and cells are undergoing rapid cell division. It has been more difficult to accept, however, that environmental agents could affect DNA methylation patterns throughout life well after tissues and organs are formed and their methylation pattern is established. The model proposed here offers a possible mechanism for alterations in methylation in adult tissue by proposing that the DNA methylation machinery remains active throughout life and thus sensitive to, for example, the social environment and the effects of diet. Two relatively recent lines of data provide some support for

this hypothesis. One line of evidence comes from our study of epigenetic programming of stress responses by maternal care (Meaney and Szyf, 2005a). Another line of evidence comes from the role of nutritional constituents such as methionine that reverse such epigenetic alterations, and that influence behavior and mental health.

3. Epigenetic programming of the stress response: the role of maternal behavior and diet

3.1. *Maternal care as an epigenetic regulator of the stress response*

In the rat, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (i.e., High LG mothers) over the first week of life show increased hippocampal GR expression, enhanced glucocorticoid feedback sensitivity, decreased hypothalamic corticotrophin releasing factor expression, and more modest HPA stress responses compared to animals reared by Low LG mothers (Francis et al., 1999; Liu et al., 1997). Cross-fostering studies suggest direct effects of maternal care on both gene expression and stress responses (Francis et al., 1999; Liu et al., 1997). These studies support an epigenetic mechanism, since the fostering mother and not the biological genetic mother define the stress response of its adult offspring. We have demonstrated that, for example, the GR exon 1_7 promoter is programmed differently in the hippocampus of offspring of the High and Low LG mothers and that differences which emerge between day 1 and 8 after birth remain stable thereafter. These differences include histone acetylation, DNA methylation, and the occupancy of the promoter with the transcription factor NERVE GROWTH FACTOR-INDUCIBLE PROTEIN A (NGFI-A) (Weaver et al., 2004). A comprehensive analysis of the hippocampal transcriptome of the adult offspring of High and Low LG mothers revealed differences in a few hundred genes (Weaver et al., 2005). This suggests a change in epigenetic programming in the brain of the offspring as a consequence of maternal care.

This programming by maternal behavior is stable and long lasting, but as will be discussed in the next section, is reversible by agents that interfere with either the methylation or histone deacetylation machinery (Weaver et al., 2004; 2005). Thus, the maternal care model typifies the first principles of epigenetic programming, which are stability and relative plasticity.

3.2. *Epigenetic programming by maternal care is reversible in adulthood*

The idea that epigenetic programming can be reversible in adulthood depends upon the assumption that the enzymatic machineries required to generate new methylation pattern are present in adult tissue. There is evidence from studies in cultured cells that this is indeed the case. TSA induces replication-independent demethylation in cell culture (Cervoni and Szyf, 2001). TSA induces histone acetylation by inhibiting HDACs (Yoshida et al., 1990) and thus tilting the histone acetylation equilibrium toward acetylation. We proposed that this open chromatin structure induced by hyperacetylation facilitated the interaction of demethylases with methylated DNA and thus tilted the DNA methylation equilibrium toward demethylation (Cervoni and Szyf, 2001). We therefore addressed the question of whether the epigenetic programming early in life could be modulated during adulthood.

We injected the HDACi TSA into the brain to test the hypothesis that the machineries required for the modulation of chromatin and DNA methylation were found in neurons and associated with the GR exon 1_7 promoter and that the epigenetic state was an equilibrium of modifying and demodifying enzymes. TSA injected into brains of adult offspring of Low LG maternal care increased acetylation, reduced methylation, activated GR exon 1_7 promoter to levels indistinguishable from adult offspring of High LG maternal care and reduced stress responsivity to the levels of offspring of High LG (Weaver et al., 2004).

We similarly reasoned that if the DNA methylation and chromatin state is in a dynamic equilibrium even in adult neurons, it should be possible to revert the epigenetic programming in the other direction toward increased methylation, leading to a reversal of the maternal programming of GR expression and HPA responses to stress. We therefore injected methionine, the precursor of SAM, into the brain of the adult offspring of different maternal care mothers. Dietary methionine is converted by methionine adenosyltransferase into SAM (Cantoni, 1975; Mudd and Cantoni, 1958), which serves as the donor of methyl groups for DNA methylation. SAM was shown to inhibit active demethylation (Detich et al., 2003b) and to stimulate methylation (Pascale et al., 1991). Importantly, the synthesis of SAM is dependent on the local availability of methionine (Cooney, 1993).

Methionine treatment has been previously shown to increase SAM and DNA methylation levels in the brain (Guidotti et al., 2007; Tremolizzo et al., 2002). Adult offspring of High and Low LG mothers were infused into the lateral ventricles with methionine (100μg/ml) or saline vehicle once a day for 7 consecutive days. Methionine treatment of the offspring of High LG mothers changed the DNA methylation state of GR exon 1_7 promoter and expression of GR in the hippocampus as well as increasing their stress responsiveness and reducing the time that these animals spent in the center of an open field, a measure of anxiety (Weaver et al., 2005; 2006).

An important question here is whether the effects of methionine are limited to a subset of genes such as GR or whether they disrupted the DNA methylation patterns across the entire genome. Surprisingly, results from gene expression microarray analysis performed on hippocampal tissue from a separate cohort of methionine-treated High and Low LG offspring showed that the methionine treatment significantly affected only 300 genes, representing 1% of the population of genes on the chip (Weaver et al., 2006). These findings suggest an impressive level of specificity. Several of the modified genes are relevant for the effects observed on the stress response, however it would appear that these results do not emerge as a function of a widespread alteration in hippocampal gene expression. Our findings suggest that alterations of cytosine methylation in the adult brain through global procedures are surprisingly specific. Because methionine alone does not methylate DNA but is converted to the methyl donor SAM in the DNA methylation reaction, the DNMTs must be poised to methylate GR exon 1_7 promoter. Taken together,

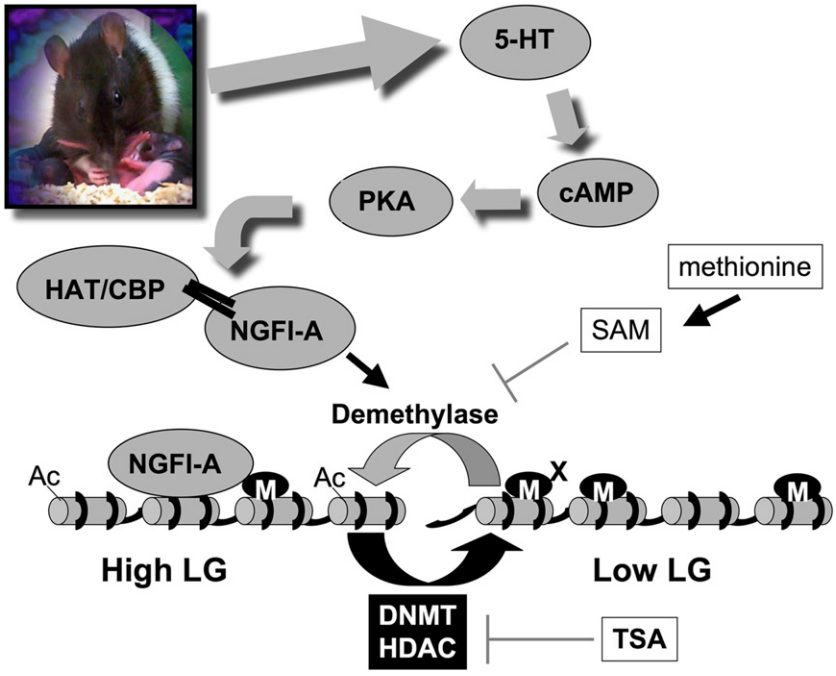

Fig. 2 – Epigenetic reprogramming by maternal care; a model. Maternal licking and grooming (LG) in the rat triggers activation of 5-HT receptors in the hippocampus leading to increased intracellular cAMP, activation of the transcription factor NGFI-A and recruitment of the HAT CBP to the GR exon 1_7 promoter. Acetylation of histone tails facilitates demethylation. In offspring of Low LG mothers, this process is reduced in comparison with offspring of High LG mothers, leading to differential epigenetic programming of the GR promoter. In the adult rat, the epigenetic state is reversible. TSA, an HDAC inhibitor, increases histone acetylation and facilitates demethylation and epigenetic activation of the gene in the offspring of the Low LG mothers. Conversely, injection of methionine to adult offspring of the High LG mothers leads to increased SAM, inhibition of demethylation, increased DNA methylation, and reduced activity of the GR exon 1_7 promoter.

the TSA and methionine experiments support the basic hypothesis that epigenetic programs in the brain are maintained by a dynamic equilibrium of methylating and demethylating enzymes, a balance which could be shifted by agents which either inhibit demethylases or stimulate DNMTs. Thus, despite the remarkable stability of epigenetic programs they are nevertheless reversible (Fig. 2).

One open question regarding the role of maternal care and methionine concerns the regional specificity of these epigenetic effects. Although the hippocampus was the focus of the aforementioned studies, other brain regions are also likely targets of these effects. The particular neural pathways influenced, however, likely depend on the brain region in question. For example, variations in maternal care are associated with altered estrogen receptor alpha expression (Champagne et al., 2003) and altered methylation of estrogen receptor alpha promoter in the hypothalamus (Champagne et al., 2006). More studies are needed to determine whether epigenetic variations of the $GR1_7$ splice variant account for reported differences in GR expression in brain areas in addition to the hippocampus.

3.3. *Mechanisms linking maternal care and epigenetic reprogramming*

We have started to decipher the molecular events which link maternal licking and grooming and epigenetic changes at the GR gene locus. In vivo and in vitro studies suggest that maternal LG or postnatal handling, which increases maternal LG, increases GR gene expression in the offspring through a thyroid hormone-dependent increase in serotonin (5-HT) activity at 5-HT7 receptors, and the subsequent activation of cyclic adenosine 30, 50 monophosphate (cAMP) and cAMP-dependent protein kinase A (PKA) (Laplante et al., 2002; Meaney et al., 1987; 2000). Both the in vitro effects of 5-HT and the in vivo effects of maternal behavior on GR mRNA expression are accompanied by increased hippocampal expression of the NGFI-A transcription factor. The GR exon 1_7 promoter region contains at least one binding site for NGFI-A (McCormick et al., 2000). Interestingly, NGFI-A was previously shown to regulate transcription of the transcriptional coactivator and histone acetyl transferase CBP by both repression and activation under different cellular challenges (Yu et al., 2004). Signaling pathways that result in increased cAMP also activate CBP (Chawla et al., 1998). NGFI-A and CBP are recruited to the GR exon 1_7 promoter in response to maternal care, which explains the increased acetylation and demethylation observed in offspring of High LG mothers (Weaver et al., 2007). Tissue culture experiments demonstrate that recruitment of NGFI-A to the GR exon 1_7 promoter results in replication-independent DNA demethylation. The recruitment of NGFI-A to the promoter facilitates the interaction of MBD2, as mentioned above a protein proposed to be involved in replication-independent DNA demethylation, with the

promoter (Weaver et al., 2007). Further experiments are required, including specific knock down of NGFI-A, CBP and MBD2 in vivo to fully demonstrate the pathway linking exposure to maternal care and demethylation of specific loci. Nevertheless, these experiments chart a feasible route leading from a behavioral exposure to a chemical change in chromatin (Fig. 2).

3.4. *Dietary contributions to DNA methylation and histone modifications*

The experiments described above involving infusion of methionine into the lateral ventricles of the brain raise the possibility that diet can affect the phenotype being studied. Because intracellular levels of methionine can be affected by both dietary intake and polymorphisms of enzymes involved in methionine metabolism, such as methylenetetrahydrofolate-reductase (Friso et al., 2002), it is tempting to consider the possibility that diet could modify epigenetic programming in the brain not only during early development but also in adult life.

Human epidemiological and animal model data indicate that susceptibility to adult-onset chronic disease is influenced by persistent adaptations to prenatal and early postnatal nutrition (Lucas, 1998). Rodent models have been particularly useful in elucidating the mechanisms involved in these developmental effects. For example, in rats, dietary L-methionine has been shown to be crucial for normal brain development, brain aging, and the pathogenesis of neurodegenerative disorders, playing an essential role in gene expression, protein synthesis, cell signaling, lipid transport/metabolism, and neuron survival (Slyshenkov et al., 2002; Van den Veyver, 2002). DNA methyltransferase requires SAM to establish or maintain DNA methylation patterns. Synthesis of SAM is dependent on the availability of dietary folates, vitamin B12, methionine, betaine, and choline (Cooney, 1993). Developmental choline deficiency alters SAM levels and global and gene-specific methylation (Kovacheva et al., 2007; Niculescu et al., 2006), and prenatal choline availability has been shown to impact neural cell proliferation and learning and memory in adulthood in rodents (Glenn et al., 2007; Meck et al., 1989; Meck and Williams, 2003). Several studies have shown that additional dietary factors, including zinc and alcohol, can influence the availability of methyl groups for SAM formation, and thereby influence CpG methylation (Davis and Uthus, 2004; Pogribny et al., 2006; Ross, 2003; Ross and Milner, 2007). Maternal methyl supplements affect epigenetic variation and DNA methylation and positively affect health and longevity of the offspring (Cooney et al., 2002; Waterland and Jirtle, 2003; Wolff et al., 1998). We hypothesize that reversal of epigenetic states in the brain, such as the remethylation of the exon 1_7 GR promoter, could be triggered not only by pharmacological agents but also by stable variations in environmental conditions.

Other studies have shown that certain dietary components may act as an HDACi, including diallyl disulfide, sulforaphane, and butyrate (Dashwood et al., 2006). For example, broccoli, which contains high levels of sulforaphane, has been associated with H3 and H4 acetylation in peripheral blood mononuclear cells in mice 3–6h after consumption (Dashwood and Ho, 2007). The long-term consequences of such epigenetic effects on human health remain to be studied, however HDACis are an active area of research as anti-inflammatory and neuroprotective agents in autoimmune diseases such as lupus and multiple sclerosis (Gray and Dangond, 2006), and sodium butyrate has been shown to have antidepressant effects in mice (Schroeder et al., 2007). Thus, it is conceivable that dietary compounds that influence histone acetylation may affect signaling mechanisms that regulate neural function. In light of the aforementioned link between histone modifications and DNA methylation, future studies are needed to address the possibility that sustained exposure to such compounds may affect DNA methylation at susceptible loci, with implications for mental health in humans. Further studies are required to map the effects of dietary components on epigenetic programming. The advent of whole-genome mapping methodologies will allow a detailed definition of the impact of dietary variations at different stages in life on long-term epigenetic programming.

4. Epigenetic contributions to mental health

The questions raised by evidence that epigenetic changes result in stable long-term changes in gene function that may nevertheless be reversible have broad ranging implications for our understanding of social, physiological and pathological processes and their interrelationships. In humans, several of the questions are similar to those raised by experimentation in non-human animals reviewed above, while others may have particular relevance to human populations. For example, what is the evidence for and the magnitude of inter-individual differences in the epigenetic profiles in humans, particularly in genomic loci involved in behavior? Could differences in early life adversity have long-term effects on epigenetic processes in humans, including increased risk for psychopathology? Finally, can behaviorally-mediated epigenetic reprogramming alter and be altered in response to diet? This list is by no means exhaustive and will serve in the following discussion only to illustrate particular ways in which these challenges are beginning to be addressed.

4.1. *Interindividual differences in DNA methylation in humans*

One line of evidence supporting the concept that there is a lifelong drift in DNA methylation in normal somatic tissue comes from the hypermethylation observed in aging tissue (Ahuja et al., 1998; Issa, 2000). Similarly, a recent study of monozygotic twins has revealed that a difference in DNA methylation emerges later in life, suggesting an environmental rather than a genetic basis for the lifelong DNA methylation drift (Fraga et al., 2005). The dynamic plasticity of the DNA methylation patterns revealed by these studies and its responsiveness to both the chemical and behavioral environment raises the possibility that errors in DNA methylation might emerge during adulthood and lead to changes in gene expression and the emergence of late onset pathologies (Feinberg, 2007).

4.2. *Influence of DNA methylation on mental health*

Genetic defects in genes encoding the DNA methylation and chromatin machinery exhibit profound effects on mental health. A classic example is RETT syndrome, a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in females which is caused by mutations in the methylated DNA binding protein MeCP2 (Amir et al., 1999). Mutations in MeCP2 and reduced MeCp2 expression were also associated with autism (Ben Zeev Ghidoni, 2007; Herman et al., 2007; Lasalle, 2007; Nagarajan et al., 2006). ATRX a severe, X-linked form of syndromal mental retardation associated with alpha thalassaemia (ATR-X syndrome) is caused by a mutation in a gene which encodes a member of the SNF2 subgroup of a superfamily of proteins with similar ATPase and helicase domains which are involved in chromatin remodeling (Picketts et al., 1996). The ATRX mutation is associated with DNA methylation aberrations (Gibbons et al., 2000). Although these genetic lesions in the methylation machinery were present through development and are thus fundamentally different from methylation changes after birth, these data nevertheless support the hypothesis that DNA methylation defects could lead to mental pathologies as well. Thus, it is possible that environmental exposures which would affect the activity of the methylation machinery would also lead to behavioral and mental pathologies.

There are some data indicating aberrant methylation in late onset mental pathologies, although it is unclear whether these changes in DNA methylation originated during embryogenesis or later in life as a response to an environmental exposure. The gene encoding *REELIN*, a protein involved in neuronal development and synaptogenesis, which is implicated in long-term memory, was found to be hypermethylated in brains of schizophrenia patients. The methylation of *REELIN* was correlated with its reduced expression and increased DNMT1 expression in GABAergic neurons in the prefrontal cortex (Chen et al., 2002; Costa et al., 2002; 2003; Grayson et al., 2005; Veldic et al., 2007).

The promoters of the genes encoding rRNA were found to be heavily methylated in hippocampi from subjects who committed suicides relative to controls (McGowan et al., 2008). Methylation of rRNA defines the fraction of rRNA molecules which are active in a cell, and the output of rRNA transcription defines to a large extent the protein synthesis capacity of a cell (Brown and Szyf, 2007). Protein synthesis is critical for learning and memory. Thus, a reduced capacity for protein synthesis required for learning and memory in brains of suicide victims could be epigenetically determined. This might be involved in the pathology leading to suicide. Thus, evidence is emerging that aberrant DNA methylation is involved in psychopathologies. Because it remained unclear whether the documented epigenetic aberrations were present in the germ line or whether they were truly late onset changes, we examined the genomic and anatomical specificity of rRNA methylation. We found that the sequence of rRNA was identical in all subjects, and there was no difference in methylation between suicide victims and controls in the cerebellum, a brain region not normally associated with psychopathology, nor were there genome-wide differences in levels of methylation (McGowan et al., 2008). These data imply that epigenetic effects that influence psychopathology likely target particular neural pathways.

4.3. *Chromatin modification and its role in mental health*

The fact that histone methylation is reversible provides a wide platform for pharmacological and therapeutic manipulations of the state of histone methylation in both directions. Both histone demethylases and histone methyltransferase are excellent candidates for new drug discovery. Understanding the intricate details of their genomic targets will allow the design of targeted and specific therapeutics.

The epigenetic effects of current clinically used monoamine oxidase inhibitors provide leads for the further development of therapies targeting the epigenome. For example, H3-K4Me2 is a hallmark of active genes and the target of the histone demethylase LSD1 which demethylates H3-K4Me2. Interestingly, certain non-selective monoamine oxidase inhibitors used as antidepressants such as Tranylcypromine that were clinically used for some time and believed to be acting on monoamine oxidases also appear to inhibit LSD1 demethylase (Lee et al., 2006). It is tempting to speculate that inhibition of LSD1 is part of the mechanism of action of these antidepressants through activation of critical genes suppressed by the H3-K4Me2 demethylating activity of LSD1 in the brain (Shi et al., 2004) or by repressing genes activated by the H3-K9Me2 demethylation activity of LSD1 (Metzger et al., 2005). Thus, it is possible that LSD1 inhibition is involved in the mechanism of action of antidepressive agents. It is tempting to speculate that selective inhibitors of LSD1 might be effective as antidepressants. This is an idea that might be pursued in the future.

Chromatin acetylation and memory were shown to be impaired in CBP knock out mice, suggesting a role for acetylation in memory formation (Alarcon et al., 2004). The fact that valproic acid, a long established antiepileptic and mood stabilizer, is also an HDACi (Phiel et al., 2001) alluded to a possible role for HDACi in treating mental disorders such as schizophrenia and bipolar disorder. Valproic acid has some effect in alleviating psychotic agitation as an adjunct to antipsychotics in schizophrenia (Bowden, 2007; Yoshimura et al., 2007). HDACi were shown to improve memory and induce dendritic sprouting in a transgenic mouse model of neurodegeneration, suggesting that HDACi might be of use in treating neurodegeneration and memory loss as well (Fischer et al., 2007). Although biological and behavioral effects of HDACi in the brain are somewhat characterized, the specific gene targets of HDACi in the brain and their function in mental pathologies are not well delineated. Nevertheless, the limited clinical and animal data suggest a potentially important role for HDACi in treatment of mental disorders. Experiments with a novel HDACi from the benzamide class N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)aminomethyl]benzamide derivative (MS-275) in mice resulted in brain region specific induction of acetylation in the frontal cortex at two genes involved with schizophrenia pathogenesis, *REELIN* and *GAD(67)* (Simonini et al., 2006). Valproic acid was shown to induce the expression of *REELIN*, which was silenced by methionine treatment in mice (Dong et al., 2007). These studies raise the possibility that treatment of schizophrenics

with HDACi might cause activation of expression of critical genes such as *REELIN* and could reverse the course of this disease (Sharma et al., 2006). Several clinical trials have tested valproate as an adjunctive therapy to antipsychotics in schizophrenia (Basan and Leucht, 2004; Bowden, 2007; Citrome et al., 2007). There are indications that valproate might improve violent episodes in a subset of schizophrenia patients (Basan and Leucht, 2004) and might have an effect on euphoric mania in combination with antipsychotics (Bowden, 2007) as well as features of manic symptomatology in bipolar disorders (Bowden, 2007). It should be noted that many of these trials were of small size and that further clinical trials are needed with valproate and with more potent and selective HDACi to methodically test the therapeutic potential of HDACi in mental pathologies.

One question that needs to be addressed is whether the observed defects in histone acetylation in mental disease are a consequence of aberrant deregulation of the overall levels of certain HDAC isotypes or HATs, or whether it involves the aberrant targeting of HDAC to a selection of promoters. The fact that inhibition of a general enzyme such as HDAC results in exquisite positive changes in the brain implies some specificity, even for a general inhibitor of a specific class of HDACs as discussed above. How could such specificity be achieved by treatment with non-selective HDACi? It will be important to delineate the response of the transcriptomes of different brain regions to HDACi and to map the genes that are critically involved in the molecular pathology of the disease. It will also be important to characterize the critical isoforms of HDAC for regulation of these genes. The advent of isotypic specific HDACi might enhance the efficacy and potency of the treatment and reduce its toxicity.

4.4. *Relevance of diet to the risk for psychopathology*

The idea that epigenetic modifications play a role in cancer has gained wide acceptance over the last two decades (Szyf, 2008). There has been more recent acknowledgement that metabolic syndrome has an epigenetic component (Ross and Milner, 2007). Evidence that nutrition plays a role at the interface between the environment and the genome in cancer and metabolic syndrome is beginning to be recognized. However, there is as yet little evidence for the role of nutrition in the epigenetic regulation of mental health. As mentioned above, a wide range of epigenetic effects influence the epigenetic status of the brain, and some nutritional components such as SAM and sulfophrane can mitigate changes in DNA methylation and chromatin structure akin to those observed by classical drugs used to treat psychopathology, such as valproic acid and the monoamine oxidase inhibitors. It is interesting to speculate that nutritional components, especially those to which humans are exposed developmentally or via sustained exposure – particularly to those which act to modify chromatin – will have effects on mental health and risk for psychopathology.

The possible involvement of DNA methylation in schizophrenia implies that pharmacological and nutritional agents which increase SAM levels in the brain might aggravate schizophrenia. For example, methionine treatment was shown in the 60s to aggravate schizophrenia (Brune and Himwich, 1962; Israelstam et al., 1967). Similarly, there might be questions raised as to the impact of folate supplementation during pregnancy and beyond. Folates are required for the synthesis of tetrahydrofolate, which is required for methionine synthesis and consequently SAM levels. Research combining the identification of polymorphisms associated with folate metabolism with psychopathology may identify effects of methionine synthesis on risk for schizophrenia (Muntjewerff and Blom, 2005).

Another of the possible interactions between dietary components that modify the DNA methylation machinery and effects on mental health in humans may be found in the effects of SAM in mood disorders (McGowan and Kato, 2008). Many studies have found SAM to have antidepressive effects (Papakostas et al., 2003). Interestingly, in one study, nine of 11 patients with bipolar depression treated with SAM switched to mania, suggesting a specific effect of SAM on bipolar depression (Carney et al., 1989). As mentioned above, central infusion of L-methionine, a precursor of SAM, increases DNA methylation of the promoter of the GR gene. Methionine treatment was found to abolish the effect of a High LG mother on the offspring, leading to increased DNA methylation of GR and exacerbating a measure of behavioral despair (Weaver et al., 2005). The fact that SAM, which similarly enhances DNA methylation, is effective in the treatment of depression is apparently contradictory to this effect of methionine. However, SAM is a methyl residue donor not only for the DNA methylation reaction but also for other enzymatic reactions. For example, creatine is produced from SAM and guanidinoacetate, and SAM treatment increases phosphocreatine levels in the brain (Silveri et al., 2003). This effect may also contribute to the antidepressive effect of SAM because decreased phosphocreatine levels have been reported in bipolar depression (Kato et al., 1994). It is becoming clear that we need to consider these issues in the future when assessing the safety of drugs, nutraceuticals and dietary habits, as DNA methylation in the brain has both pharmacological and toxicological implications.

5. Summary and prospective

The realization that the genome is programmed by the epigenome and that this programming might be as important as the sequence itself in executing genome functionality offers a new approach to the long-standing mystery of gene-environment interactions. Epigenetic aberrations might have similar consequences to genetic damage, as far as gene expression and the resulting phenotype are considered. Epigenetic marks, though potentially reversible, are stable and could be long lasting. The differential epigenetic status of the GR exon 1_7 promoter in the offspring of High LG mothers is a possible mechanism for the maternal effect on hippocampal GR expression and HPA responses to stress. These findings provide a possible mechanism for the 'environmental programming' of gene expression and function during development and beyond. Studies on the reversal of maternal effects on DNA methylation using either TSA or methionine suggest that neurons express the enzymatic machinery necessary for methylation and demethylation in adulthood. DNA

methylation, although a stable epigenetic mark maintained through carbon–carbon bonds, can be altered through sustained alterations of chromatin structure such as histone acetylation. These findings thus raise the fascinating question of the degree to which such processes might remain sensitive to environmental regulation throughout life. The emerging understanding that late onset diseases might have an epigenetic origin points to the importance of developing screens to identify epigenetic chemoprotective agents present in our diet.

Perhaps one of the finest examples of how the epigenome mediates the effects of the environment on our genome comes from studies of endocrine disruptors [for a review see Jirtle and Skinner (2007)]. Endocrine disruptors cause epigenetic changes by DNA methylation, which are heritable in rodents and can promote disease across subsequent generations (Anway et al., 2005). These observations put forward the thought-provoking notion that environmental exposures in one generation could have an impact on phenotype and disease susceptibility on generations to come. Interestingly, exposure to endocrine disruptors affect female mate preference in rodents three generation removed from the exposure, raising the possibility that epigenetics is a yet unappreciated force in evolution (Crews et al., 2007). In addition, dietary manipulations that affect the availability of the methyl donors during development had a protective effect against endocrine disruption (Dolinoy et al., 2007). New data from behavioral studies is shedding light on the relationship between the social environment and epigenetic programming. It has also illustrated the potential lifelong dynamic nature of the epigenome. The relationship between behavior and the epigenome is bilateral; behavior could result in epigenetic programming and epigenetic programming could affect behavior. Similarly behavior might affect susceptibility to dietary preferences while dietary preferences might have a long-term effect on behavior through affecting epigenetic reprogramming. In humans, such effects might contribute to the risk for and resilience to psychopathology.

Another important principle that is emerging from these studies is that behavioral parameters should be taken into consideration in our analysis of the environmental impact on the epigenome. The dynamic equilibrium of DNA methylation provides a template for diet to act upon. Dietary components could act through cellular signaling pathways, leading from cell surface receptors down to transacting factors, that deliver chromatin-modifying enzymes to specific sequences. The dynamic epigenome has obviously adaptive and physiological roles in the crosstalk between our environment and our inherited genome, but could at the same time serve as a target for dietary components (Figs. 1–2). Unraveling the conduits between our diet and our genomes should have an important impact on our health.

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