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Epigenetic Mechanisms for the Early Environmental Regulation of Hippocampal Glucocorticoid Receptor Gene Expression in Rodents and Humans

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Parental care influences development across mammals. In humans such influences include effects on phenotypes, such as stress reactivity, which determine individual differences in the vulnerability for affective disorders. Thus, the adult offspring of rat mothers that show an increased frequency of pup licking/grooming (ie, high LG mothers) show increased hippocampal glucocorticoid receptor (GR) expression and more modest hypothalamic–pituitary–adrenal responses to stress compared with the offspring of low LG mothers. In humans, childhood maltreatment associates decreased hippocampal GR expression and increased stress responses in adulthood. We review the evidence suggesting that such effects are mediated by epigenetic mechanisms, including DNA methylation and hydroxymethylation across GR promoter regions. We also present new findings revealing associated histone post-translational modifications of a critical GR promoter in rat hippocampus. Taken together these existing evidences are consistent with the idea that parental influences establish stable phenotypic variation in the offspring through effects on intracellular signaling pathways that regulate the epigenetic state and function of specific regions of the genome.

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INTRODUCTION

The quality of family life influences the development of individual differences in vulnerability for multiple forms of mental illness, including affective illnesses. As adults, victims of childhood physical or sexual abuse or parental neglect are at considerably greater risk for affective disorders (eg, Bifulco *et al*, 1991; Brown and Anderson, 1991; McCauley *et al*, 1997; Felitti *et al*, 1998; Shonkoff *et al*, 2009). These findings were confirmed in a prospective, longitudinal study that confirms the link between abuse/neglect and depression (Widom *et al*, 2007). Moreover, childhood maltreatment also associates with an increased severity of illness, reduced treatment responsivity, and increased comorbidity (Widom *et al*, 2007). Broader forms of familial dysfunction including persistent emotional and physical neglect, family conflict, and conditions of harsh, inconsistent discipline compromise cognitive and emotional development (Ammerman *et al*, 1986; Trickett and McBride-Chang, 1995; Repetti *et al*, 2002; Lupien *et al*, 2009) and increase the risk for depression and anxiety disorders (Holmes and Robins, 1987, 1988; Gottman, 1998; Hill *et al*, 2001) to a level comparable to that for abuse. More subtle relationships exist. Low scores on measures of parental bonding, reflecting cold, distant parent–child relationships, particularly low maternal care, are associated with a significantly increased risk of depression and anxiety in later life (eg, Canetti *et al*, 1997; Parker, 1981; Kendler *et al*, 2002; Hill *et al*, 2000). And again, the risk is not unique to mental health. Russak and Schwartz (1997) found that by midlife, those individuals who, as undergraduate students, rated their relationships with parents as cold and detached had a fourfold greater risk of chronic illness, including depression and alcoholism, as well as heart disease and diabetes. Family life also serves as a source of resilience in the face of chronic stress (Rutter, 1979). Thus, warm, nurturing families tend to promote resistance to stress and to diminish vulnerability to stress-induced illness (Smith and Prior, 1995; Repetti *et al*, 2002). The epidemiology of affective disorders reflects the profound influence of family life on neural development and mental health.

Parental factors also serve to mediate the effects of adversity derived from extra-familial sources on neurodevelopment

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(Hackman *et al*, 2010). For example, the effects of poverty on emotional and cognitive development are mediated by parental factors to the extent that if such factors are controlled, there is no discernible effect of poverty on child development (Conger and Donnellan, 2007; McLloyd, 1998; also see Linver *et al* (2002)). Treatment outcomes associated with early intervention programs are routinely correlated with changes in parental behavior: In cases where parental behavior proves resistant to change, treatment outcomes for the children are seriously limited. The effects of intervention programs that directly target parent–child interactions on endophenotypes associated with affective disorders (eg, Belsky, 1997; Olds *et al*, 1998; Klein Velderman *et al*, 2006; Van Zeijl *et al*, 2006) provide evidence for the causal influence of parenting on mental health.

THE BIOLOGY OF PARENTAL INFLUENCES

A critical question concerns the mechanisms that mediate the enduring parental influence on the health of offspring. The relationship between social influences over development and health in adulthood appears to be, in part, mediated by the development of individual differences in neural systems that underlie the expression of behavioral and endocrine responses to stress (Seckl and Meaney, 1994; Nemeroff, 1996; Sroufe, 1997; Francis *et al*, 1999; Francis *et al*, 1999a; Repetti *et al*, 2002; Fish *et al*, 2004; Klaassens *et al*, 2009; Cichetti *et al*, 2010). Thus, physical and sexual abuse in early life increases endocrine and autonomic responses to stress in adulthood (DeBellis *et al*, 1994; Heim *et al*, 2000). Likewise, variations in parental care associate with individual differences in neuroendocrine and autonomic responses to stress in humans (Flinn and England, 1995; Leucken, 1998, 2000; Pruessner *et al*, 2004; Taylor *et al*, 2004; Taylor *et al*, 2006), as well as emotional reactivity (Reid and Crisafulli, 1990; and see Repetti *et al* (2002)). Finally, there is considerable evidence in favor of the hypothesis that individual differences in stress reactivity associate with the risk for depression (Wichers *et al*, 2007, 2009). Thus, the influence of familial depressive illness is, in part at least, mediated by increased stress reactivity, enhancing the response of the individual to mild, regular stressors (ie, hassles).

Parental effects occur across a variety of species from plants to mammals (Meaney, 2001; Cameron *et al*, 2005; Maestripieri and Mateo, 2009). Such effects imply an enduring influence of environmental signals operating during early development on genome function. We explore the potential mechanisms for such parental effects examining the influence of variations in maternal care in the rat on the development of individual differences in behavioral and endocrine responses to stress. Lactating female Long-Evans rats (an out-bred strain of *rattus norvegicus*) exhibit considerable variation in the frequency of pup licking/grooming (LG; Champagne *et al*, 2003). Individual differences in the frequency of pup LG among adult female rats are reliable across multiple litters, and thus a stable feature of the maternal phenotype. We use observational procedures to define mothers that consistently show high or low levels of pup LG (ie, high *vs* low LG mothers). Variations in pup LG over the first week of postnatal life rat affect the development of behavioral and hypothalamic–pituitary–adrenal (HPA) responses to stress in adulthood (Liu *et al*, 1997; Caldji *et al*, 1998; Francis *et al*, 1999; Menard *et al*, 2004; Weaver *et al*, 2004, 2005; Zhang *et al*, 2006; Toki *et al*, 2007). Behavioral responses to environmental stressors include a cessation of exploration or appetitive behavior (Caldji *et al*, 1998; Francis *et al*, 1999; Weaver *et al*, 2006; Toki *et al*, 2007), as well as active attempts to escape from threat (Menard *et al*, 2004). For example, in a novely-induced suppression of feeding test in which food-deprived animals are provided food in a novel context, the adult offspring from high LG mothers show a shorter latency to begin eating and eat for a longer period of time (Caldji *et al*, 1998; O'Donnell, unpublished observation). The offspring of low LG mothers also show increased vulnerability for stress-induced learned helplessness (Kurata *et al*, 2009).

Likewise there are differences in HPA responses to acute stress apparent in both circulating levels of pituitary adrenocorticotropin (ACTH) and adrenal corticosterone. As adults, the offspring of high LG mothers show more modest plasma ACTH and corticosterone responses to acute stress in comparison with animals reared by low LG mothers (Liu *et al*, 1997; Weaver *et al*, 2004, 2005; Champagne *et al*, 2003; Toki *et al*, 2007; Kurata *et al*, 2009). Circulating glucocorticoids act at glucocorticoid receptor (GR) sites in corticolimbic structures, such as the hippocampus, to regulate HPA activity. Such feedback effects target corticotropin releasing factor (CRF) synthesis and release at the level of the paraventricular nucleus of the hypothalamus (PVNh). The offspring of high LG mothers show significantly increased hippocampal GR mRNA and protein expression, enhanced glucocorticoid negative feedback sensitivity, and decreased hypothalamic CRF mRNA levels. Importantly, hippocampal infusion of a GR antagonist completely eliminates the maternal effect on HPA responses to stress, suggesting a direct relation between hippocampal GR expression and the magnitude of the HPA response to stress.

Importantly, effects of maternal care on gene expression and stress responses of the adult offspring are reversed with cross-fostering (Francis *et al*, 1999; Caldji *et al*, 2003; Weaver *et al*, 2004): stress responses of adult animals born from low LG mothers and reared by high LG dams are comparable to normal offspring of high LG mothers (and vice versa). Moreover, variations in the frequency of pup LG toward individual pups of the same mother are significantly correlated with hippocampal GR expression in adulthood (van Hasselt *et al*, 2012). These findings, as well as those from studies that directly manipulate the frequency of pup LG by the dam reveal a direct relation between maternal care and the phenotypic development of the offspring.

MOLECULAR TRANSDUCTION OF PARENTAL SIGNALS

Tactile stimulation derived from maternal licking appears to be the critical environmental signal for the regulation of hippocampal GR expression in the neonate. *In vivo* studies with rat pups or *in vitro* studies using cultured hippocampal neurons suggest that maternal effects on hippocampal GR expression are mediated by increases in hippocampal serotonin (5-HT) turnover and the expression of the nerve-growth factor-inducible factor-A (NGFI-A) transcription factor (Meaney *et al*, 2000; LaPlante *et al*, 2002; Mitchell *et al*, 1990, 1992). *In vitro*, 5-HT through the activation of a 5-HT_7 receptor increases the activity of cAMP-dependent signaling pathways in hippocampal neurons, resulting in elevated expression of NGFI-A. The effect of various 5-HT agonists on GR expression in hippocampal neurons is strongly correlated with the effect on cAMP formation. Activation of these signaling cascades leads to an increased GR expression. In cultured hippocampal neurons, the effect of 5-HT on GR expression is (1) blocked by 5-HT_7 receptor antagonists or inhibitors of protein kinase A, (2) mimicked by 5-HT_7 receptor agonists or treatments with stable cAMP analogs, and (3) eliminated by antisense or siRNA knockdown of NGFI-A mRNA (Weaver *et al*, 2007; Hellstrom *et al*, in press). *In vivo*, the effect on GR is blocked with 5-HT receptor antagonists (Mitchell *et al*, 1990, 1992). Moreover, the increase in hippocampal 5-HT activity is associated with a maternally regulated increase in the conversion of thyroxine to triiodothyronine (T3; Hellstrom *et al*, in press): T3 administration in neonatal period, which regulates the 5-HT systems activity, mimics the effects of increased pup LG on both NGFI-A expression and hippocampal GR programming (Meaney *et al*, 1987; Mitchell *et al*, 1990; Hellstrom *et al*, in press). Interestingly, the activation of ascending 5-HT systems during postnatal development also regulates the development of corticolimbic systems implicated in fear behavior (Gross *et al*, 2002; Gross and Hen, 2004).

The 5' non-coding variable exon 1 region of the hippocampal GR gene (Figure 1) contains multiple alternate promoter sequences including a neuron-specific, exon 1_7 sequence (McCormick *et al*, 2000). Increased pup LG enhances hippocampal expression of GR mRNA splice variants containing exon 1_7 sequence (McCormick *et al*, 2000; Weaver *et al*, 2004, 2007; Hellstrom *et al*, in press), which contains an NGFI-A response element (Crosby *et al*, 1991). Pup LG increases hippocampal NGFI-A expression and binding to the exon 1_7 promoter (Weaver *et al*, 2004, 2007; Hellstrom *et al*, in press). Cotransfection of an NGFI-A vector and an exon 1_7–luciferase construct shows increased luciferase activity, reflecting NGFI-A-induced activation of transcription through the exon 1_7 promoter (Weaver *et al*, 2007; Hellstrom *et al*, in press). The effect of NGFI-A is eliminated by a site-directed mutation within the NGFI-A response element of the exon 1_7 promoter (Weaver *et al*, 2007) revealing that it is the physical interaction of NGFI-A with its response element that triggers the increase in transcriptional activity. Moreover, infection of hippocampal neurons with an NGFI-A expression plasmid increases both total GR mRNA and exon 1_7-containing GR mRNA (Hellstrom *et al*, in press). A series of *in vivo* studies show that the association of NGFI-A with the exon 1_7 promoter is actively regulated by pup LG and artificially generated tactile stimulation of the pups yields the same effect (Hellstrom *et al*, in press). Thus, chromatin immunoprecitipation (ChIP) assays reveal increased binding of NGFI-A to the exon 1_7 promoter in pups of high compared with low LG mothers, but only in the period following a nursing bout with pup LG: hippocampal tissue samples obtained 20 min following a nursing bout, with no subsequent interaction between the mother and pup, do not

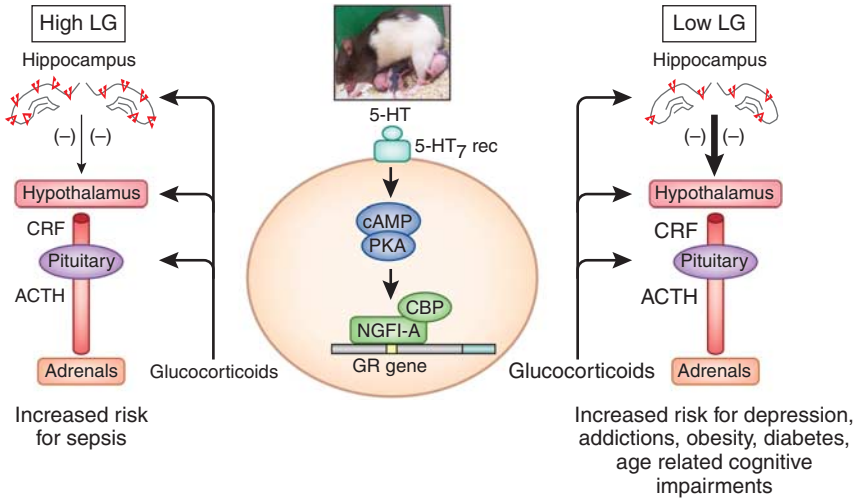

Figure 1. Glucocorticoid receptor gene organization. Schema describing the organization of the rat and human glucocorticoid receptor gene, including the 9 exon regions. Exons 2–9 code for the glucocorticoid receptor protein. Exon 1 is comprised of multiple, tissue-specific promoter regions (rat is based on McCormick *et al* (2000) and human on Turner and Muller (2005)). The rat exon 1_7 sequence shares ~70% sequence homology with the human exon 1_F sequence, and both are highly expressed in hippocampus. ACTH, adrenocorticotropin; CRF, corticotropin releasing factor.

reveal the difference in NGFI-A association. Perhaps most convincingly, artificial tactile stimulation of pups increases hippocampal NGFI-A expression and NGFI-A binding to the exon 1_7 promoter.

There is a similar effect on hippocampal *GAD1* (Zhang *et al*, 2010), an NGFI-A-regulated gene that encodes for glutamic acid decarboxylase, the rate-limiting enzyme for GABA synthesis. The association of NGFI-A with the GAD1 promoter is increased in the offspring of high compared with low LG mothers, but only following a nursing bout. Similarly, hippocampal neuronal cultures treated with 5-HT show an increase in GAD1 expression and the effect is blocked by an siRNA targeting NGFI-A. These findings suggest that maternal care regulates the expression of a range of NGFI-A-sensitive genes.

However, the critical issue concerns the mechanism by which hippocampal GR expression remains elevated following weaning and separation from the mother. One possibility is that the increased NGFI-A–exon 1_7 interaction occurring within hippocampal neurons in the pups of high LG mothers might result in an epigenetic modification of the exon 1_7 sequence that alters NGFI-A binding and maintains the maternal effect into adulthood. We focused our initial studies on potential influences on DNA methylation with the assumption that this relatively stable covalent modification was a reasonable candidate mechanism for the enduring effects of maternal care on hippocampal gene expression in the rat.

THE EPIGENETICS OF PARENTAL EFFECTS

Preliminary studies revealed greater methylation across the entire exon 1_7 GR promoter sequence in the hippocampus of adult offspring of low LG mothers. These findings suggest a parental effect on DNA methylation patterns in the offspring. More focused approaches examined the methylation status of individual CpGs in the exon 1_7 sequence using sodium bisulfite mapping. The results reveal significant differences in methylation at the 5′ CpG dinucleotide of the NGFI-A consensus sequence. This site is hypermethylated in the offspring low LG mothers, and hypomethylated in those of high LG dams. Cross-fostering reverses the differences in the methylation of the 5′ CpG site and suggests a direct relation between maternal care and DNA methylation of the exon 1_7 GR promoter (Weaver *et al*, 2004). The effect of maternal care is remarkably specific, with highly significant alterations in the methylation status of the 5' CpG, and no effect at the 3' site. Nevertheless, although less striking, there are differences in the frequency of methylation at other CpG sites on the exon 1_7 promoter.

An alternative form of DNA methylation, 5-hydroxymethylcytosine, has recently been identified, although its function is not been fully understood. Bisulfite sequencing or PCR-based approaches to the study of DNA methylation cannot distinguish between 5-methylcytosine and 5-hydroxymethylcytosine. Interestingly, the ten–eleven translocation family of enzymes can convert 5-methylcytosine to 5-hydroxymethylcytosine. 5-Hydroxymethylcytosine has been found widely distributed in embryonic stem (ES) cells, suggesting a possible function in gene regulation in ES cells. 5-Hydroxymethylcytosine has also been found enriched in certain neuronal cells (Kriaucionis and Heintz, 2009). Maternal care has a sustained effect on GRexon 1_7 DNA methylation, which cannnot exclude possible involvement of 5-hydroxymethylcytosine. We analyzed 5-hydroxymethylcytosine level in the hippocampal GRexon 1_7 promoter in rats using antibody capture (ie, 5-hydroxymethylcytosine-dependent immunoprecitipation) of hippocampal DNA. We found the level of 5-hydroxymethylcytosine of the exon 1_7 GR promoter was three times higher in hippocampal samples from the offspring of low compared with high-LG mothers (Figure 2), suggesting that the differences in DNA methylation at this site reflect, in part at least, differences in

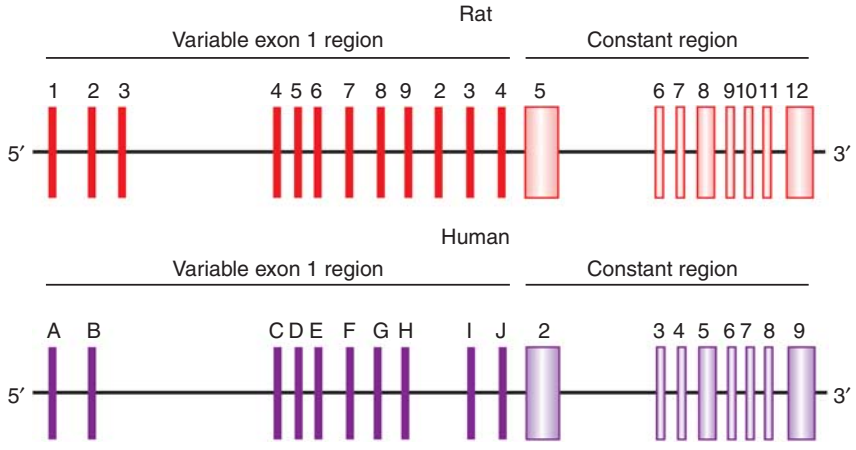

Figure 2. DNA 5-hydroxymethylcytosine (5-hmC) analysis of the exon 1_7 GR promoter. Mean ± SEM percentage levels of 5-hmC expressed as a percentage of input DNA from a 5-hmC-dependent immunoprecipitation of GR exon 1_7 promoter in hippocampal sample from adult offspring of high and low licking/grooming (LG) mothers ($n = 3$–4/group). Unmethylated and methylated controls showed negligible signal (ie, 0–3%) using a commercially available 5hmC-immunoprecipiation assay (DiagenodeCat. No. AF-104-0016). GR, glucocorticoid receptor; NGF-I, nerve-growth factor-inducible factor-A.

5-hydroxymethylcytosine. The overall level of DNA methylation is dynamic during the period of early postnatal development, a time when the differences in the frequency of pup LG between high and low LG mothers are apparent. Whether 5-hydroxymethylcytosine is also dynamic over this period and directly influenced by maternal care remains to be determined.

As DNA methylation favors a closed chromatin structure, the difference in methylation within the 5' CpG dinucleotide of the NGFI-A response element suggests alteration of NGFI-A binding to the exon 1_7 sequence. *In vitro* binding of purified recombinant NGFI-A protein to its response element using electrophoresis mobility shift assays indicate that methylation of the 5′ CpG dinucleotide in the NGFI-A response element of the exon 1_7 GR promoter inhibits NGFI-A protein binding (Weaver *et al*, 2004). Transfection studies show that (1) NGFI-A induces transcription through the exon 1_7 promoter and (2) DNA methylation of a transfected exon 1_7 construct inhibits the ability of NGFI-A to bind and activate its expression (Weaver *et al*, 2007; Hellstrom *et al*, in press). Likewise, ChIP assays indicate increased acetylated lysine 9 (K9)- histone H3 and a threefold greater binding of NGFI-A to the exon 1_7 GR promoter in hippocampal samples obtained from the adult offspring of high compared with low LG mothers (Weaver *et al*, 2004, 2005). Importantly, such differences occur despite a comparable level of hippocampal NGFI-A expression in the adult offspring of high and low LG mothers. Thus, the methylation of the 5′CpG site alters the 'affinity' of the NGFI-A consensus sequence for its ligand, resulting in a decreased level of NGFI-A binding. Finally, the sequencing involved in these studies has yet to reveal any evidence for sequence variation in this region. Thus, to our knowledge, the individual differences in GR expression in this model associates with variation at the level of epigenetic state, and not in nucleotide sequence.

The ability of DNA methylation to regulate the capacity for histone modifications, especially histone acetylation, forms a prominent link between methylation and transcription. The electrostatic bonds formed between the positively charged histone proteins and their negatively charged DNA partners demands an active chromatin remodeling process for transcriptional activation (Turner, 2001; Taverna *et al*, 2007). Chromatin remodeling is achieved through biochemical modifications of the histone proteins that control chromatin structure and thus genome function. The post-translational modifications to the histones occur through a series of enzymes that bind to the histone tails and modify the local chemical properties of specific amino acids (Shahbazian and Grunstein, 2007; Grunstein, 1997; Hake and Allis, 2006; Jenuwein and Allis, 2001; Taverna *et al*, 2007). For example, histone acetylation neutralizes the positive charge on the histone tail, opening chromatin and increasing the access of transcription factors to their DNA-binding sites. Acetylation commonly occurs at lysine residues, such as the H3K9, and is catalyzed by histone acetyltransferases and reversed by HDACs. HDACs remove

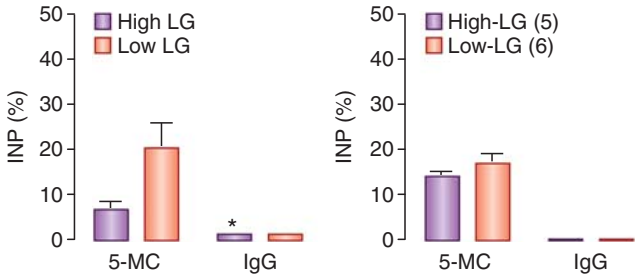

Figure 3. Histone post-translational modifications associated with the exon 17 GR promoter in rat hippocampus. Mean ± SEM levels of various histone modifications associated with the exon 17 GR promoter determined using serial micro chromatin-immunoprecipitation assays in the same hippocampal samples from adult offspring of high and low licking/grooming (LG) mothers (all antibodies form Santa Cruz). The data are expressed as a ratio of the input DNA. The middle panel reveals the correlation between levels of H3K9ac and H3K4me3.

acetyl groups from histone tails and prevent subsequent acetylation (Shahbazian and Grunstein, 2007; Szyf, 2009). Cytosine methylation attracts repressor complexes comprised of HDACs such that DNA methylation and histone acetylation are usually inversely related. H3K9ac associates with increased transcription and we found increased H3K9ac of the exon 1_7 GR promoter (Weaver *et al*, 2004, 2007; Figure 3) genes in hippocampus from the adult offspring of high compared with low LG mothers. This pattern is similar to maternal effects on hippocampal *GAD1* or *Grm1* expression; in each case decreased DNA methylation within promoter regions associates with increases in both H3K9ac and gene transcription (Zhang and Meaney, 2010; Bagot *et al*, submitted). H3K9ac tends to associate with stably transcribed regions of the genome, which is consistent with the idea of a persistent increase in hippocampal GR transcription in the adult offspring of high LG mothers.

Histone acetylation directly modifies chromatin structure through effects on the local physicochemical environment that define the chromatin state (Turner, 2001; Taverna *et al*, 2007). Additional histone modifications, notably histone methylation, influence transcription through indirect pathways that involve a complex array of transcriptional mediators (Shahbazian and Grunstein, 2007; Grunstein, 1997; Hake and Allis, 2006; Jenuwein and Allis, 2001; Bernstein *et al*, 2005; Berger, 2007; Kouzarides, 2007; Taverna *et al*, 2007). Multiple lysine and arginine residues on the histone tails are subject to methylation, which is catalyzed by distinct histone methyltransferases and reversed by histone demethylases. This process provides a signaling pathway that begins with the activation of the intracellular signals that activate the individual methylating or demethylating enzymes producing a specific epigenetic profile on the histone tails. This process links specific intracellular signals to specific histone methylation marks. The methylation profile of the histone tails is highly variable. Methylation can occur at multiple sites along the histone tails and vary in the level of methylation (mono-, di-, or tri-methylation). The resulting profile acts as a 'code' (Hake and Allis, 2006;

Jenuwein and Allis, 2001; Taverna *et al*, 2007; Zhang *et al*, 2010a) for various protein complexes that remodel chromatin and alter transcriptional activity; thus, indicating an indirect influence of histone methylation on transcription.

Certain histone modifications covary. An example of relevance here is that of H3K9ac and H3K4me. Both marks are generally present at actively transcribed regions of the genome (Ruthenburg *et al*, 2007a; Pokholok *et al*, 2005; Millar and Grunstein, 2006). Thus, we find increased H3K9ac and H3K4me3 at both regions of the exon 1_7 GR promoter and the levels of these individual marks are very highly correlated (Figure 4).

H3K4me, whether in the mono-, di-, or tri-methylated state, appears to protect CpG islands against methylation (Ooi *et al*, 2007; Thompson *et al*, 2010). Thus, genome-wide analyses reveal a negative correlation between H3K4me and CpG methylation. Interestingly, H3K4me3 appears to actively 'repel' the binding of the DNA methyltransferase, DNMT3L, which is essential for *de novo* methylation and attracts complexes containing histone acetyltransferases that open chromatin and enhance transcription factor binding (Ooi *et al*, 2007). Indeed, the absence of H3K4me3 seems to be a prerequisite for the recruitment of *de novo* D (DNMTs) and the acquisition of DNA methylation (Ooi *et al*, 2007; Thompson *et al*, 2010; Ciccone *et al*, 2009). The same relation was apparent across the exon 1_7 GR promoter, where the decreased level of DNA methylation was associated with an increased level of H3K4m3 (Figures 3 and 4). H3K4me3 targets the chromatin remodeling factor (NURF) and the Yng1 protein in the NuA3 (nucleosomal acetyltransferase of histone H3) complex to genes increasing the level of histone acetylation and transcriptional activation. This process explains the tight correlation between the levels of H3K4me3 and H3K9ac.

These findings suggest that variations in maternal care influence the methylation state of the exon 1_7 GR promoter in hippocampus, regulating NGFI-A binding, GR transcription, and HPA stress responses. The effect of CpG methylation on gene expression is, in part, mediated by the recruitment of HDAC-containing repressor complexes (Turner, 2001; Bird and Wolffe, 1999; Bird, 2001; Klose and Bird, 2006; Li, 2002; Miranda and Jones, 2007; Nan *et al*, 1998), HDAC inhibitors permit chromatin remodeling and transcription factor binding, and may thus liberate the expression of genes from methylation-induced repression. HDAC inhibition also reverses the maternal effects on hippocampal GR expression (Weaver *et al*, 2004). Chronic, central infusion of adult offspring of low LG mothers with the broad-spectrum HDAC inhibitor, trichostatin A (TSA; Weaver *et al*, 2006), significantly increased H3K9 acetylation, NGFI-A binding to the GR-1_7 promoter, and GR expression to levels comparable to those observed in the offspring of high LG mothers. TSA infusion also eliminated the effect of maternal care on HPA responses to acute stress. These results suggest a direct relation between maternal care, histone acetylation, DNA methylation of the GR-1_7 promoter, GR expression, and HPA responses to stress.

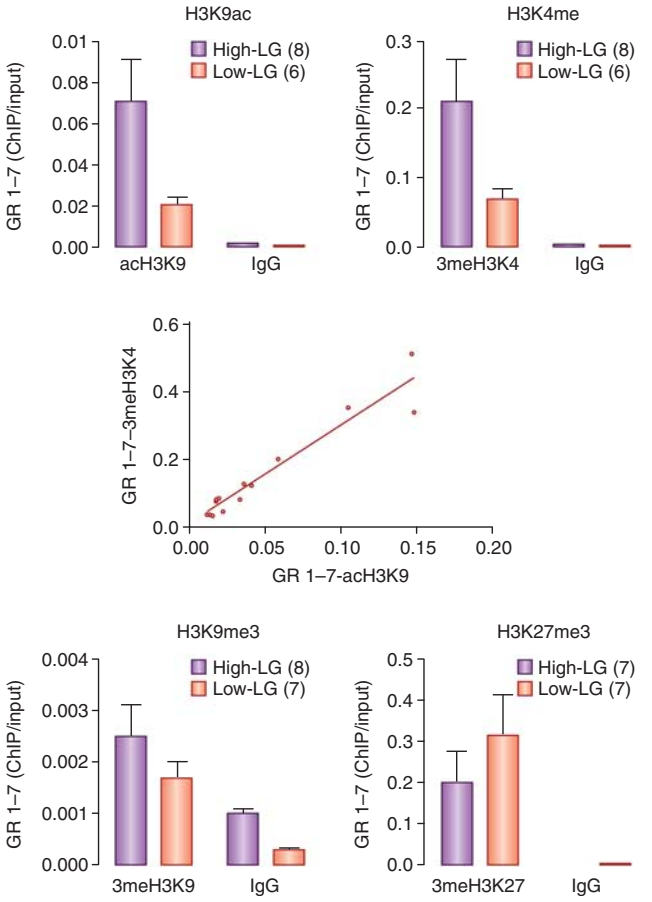

Figure 4. Histone post-translational modifications associated with the exon 17 GR promoter in rat hippocampus. Mean ± SEM levels of various histone modifications associated with the exon 17 GR promoter determined using serial micro chromatin-immunoprecipitation assays in the same hippocampal samples from adult offspring of High and Low LG mothers (all antibodies form Santa Cruz). The data are expressed as a ratio of the input DNA. The middle panel reveals the correlation between levels of H3K9ac and H3K4me3.

An obvious concern is whether the effects of maternal care on hippocampal GR expression represent a more global process in which variations in parental signals affect the methylation status of broad regions of the genome. Other studies reveal that stress-induced variations in maternal care in rat, including the frequency of pup LG, alter the methylation state of the *bdnf* gene in hippocampus (Roth *et al*, 2009). In the mouse, prolonged periods of maternal separation alter the methylation state of the promoter for the arginine vasopressin gene (AVP), increasing hypothalamic AVP synthesis and HPA responses to stress (Murgatroyd *et al*, 2009). Maternal separation in the rat associates with reduced $GABA_A$ receptor levels in the locus coeruleus (LC) and the nucleus tractus solitarius (NTS) as well as levels of the mRNA for the γ_2 subunit of the $GABA_A$ receptor complex, which confers high-affinity benzodiazepine binding in the amygdala as well as in the LC and NTS (Caldji *et al*, 2000). Both the amygdala and the ascending noradrenergic systems have been considered as critical sites for the anxiolytic effects of GABAergic inhibition. These

findings suggest that parental influences might influence the epigenetic regulation of multiple regions of the genome in different brain regions to produce a coordinated effect on the stress response of the offspring.

The NGFI-A-regulated GAD1 gene shows a similar increase in the level of promoter methylation in the hippocampus from the adult offspring of low LG mothers (Zhang *et al*, 2010). Moreover, a ChIP-chip study using high-density oligonucleotide microarrays tiling a contiguous 7 million base pair region of rat chromosome 18 containing the *NR3C1* gene at 100 bp spacing reveal coordinated alterations in H3-K9 acetylation, DNA methylation, and gene expression across a number of areas in response to variations in maternal care, including a subregion containing multiple protocadherin genes (McGowan *et al*, 2011). These results suggest a broad epigenomic response to variations in maternal care that associates with an extensive difference in gene expression.

DEVELOPMENTAL REGULATION OF HIPPOCAMPAL GR EXPRESSION IN HUMANS

A critical question is whether familial influences operating during early development in humans are linked to the stable epigenetic regulation of gene expression as in rodents. There are obvious constraints on tissue access for molecular studies of neural function in humans. These limitations are of considerable importance in the study of epigenetics mechanisms, which are potentially tissue- and even cell-type-specific. We were able to establish a translational program focusing on human hippocampus by virtue of the resources of the Québec Suicide Brain Bank (www.douglas.qc.ca/suicide). Approximately a third of individuals who die by suicide have histories of childhood adversity, including childhood sexual and physical abuse, as well as parental neglect. We (McGowan *et al*, 2009; Labonté *et al*, in press) thus showed decreased hippocampal GR expression in samples from suicide completers with histories of childhood maltreatment compared with controls (sudden, involuntary fatalities). The program is strengthened by a validated forensic interview that establishes developmental history and mental health status (Dumais *et al*, 2005; Zouk *et al*, 2006). Regression analyses across the samples showed no significant correlations between psychopathology, notably depression and substance disorders, and hippocampal GR expression. Rather the decreased hippocampal GR expression associated with a history of childhood maltreatment. There were no differences in hippocampal GR expression in samples from suicides negative for a history of childhood maltreatment. Instead, the differences in hippocampal GR expression were unique to suicide completers with a history of childhood maltreatment.

Splice variant analysis, comparable to that performed in the rat, revealed decreased expression of non-coding exons 1_B, 1_C, 1_F, and 1_H in suicides with a history of childhood maltreatment compared with both controls and suicides without a history of maltreatment. These expression differences correlated with differential DNA methylation patterns between groups in the corresponding exon 1 variant promoters. The exon 1_F sequence is of particular interest as it is the homolog of the rat exon 1_7, is highly expressed in the brain, and contains an NGFI-A response element (Turner and Muller, 2005; McGowan *et al*, 2009; Figure 1). Moreover, the exon 1_F sequence shows increased DNA methylation and decreased NGFI-A binding in samples from suicide victims with a history of maltreatment. These findings bear considerable similarity to the maternal effect in the rat and are suggestive of early-environment regulation of the neural epigenome in humans. Of interest, recent studies in independent human samples investigating the effects of early-environmental adversity on exon 1_F methylation reported consistent results (Radtke *et al*, 2011; Tyrka *et al*, 2012).

Decreased expression levels of GR exon 1_B, 1_C, and 1_H transcripts were also associated with alterations in methylation of the respective sequences, with particular sites significantly correlated with expression levels. As expected on the basis of the expression data, the exon 1_B and 1_C regions showed increased methylation at predictive sites uniquely in samples from suicide/maltreatment subjects. However, analysis of the exon 1_H GR promoter yielded an interesting profile that contrasted starkly with that observed for the other exon 1 regions (Labonté *et al*, in press). There was significantly increased DNA methylation of the exon 1_H promoter in hippocampal samples from both controls and suicide victims without a history of maltreatment by comparison with those positive for maltreatment. And the methylation of the exon 1_H promoter was positively correlated with hippocampal GR expression.

Most differentially methylated sites were found within putative transcription factors binding sites. Multiple transcription factors are predicted to bind promoters of GR non-coding exons (Turner *et al*, 2010), although, to date, only NGFI-A has been shown to activate transcription in the promoter of $GR1_F$ (Weaver *et al*, 2007; Hellstrom *et al*, in press). Nevertheless, most of the CpG sites whose methylation state were investigated in $GR1_B$, $GR1_C$, and $GR1_H$ promoters are predicted to bind transcription factors such as Sp1 and Sp3. Sp1 and Sp3 regulate GR basal expression (Nobukuni *et al*, 1995). Interestingly, there is evidence that Sp1 binding can alter the underlying methylation state of the DNA (Brandeis *et al*, 1994; MacLeod *et al*, 1994). Other known factors predicted to bind within the investigated promoter regions include NF-1, YY1, and members of the AP-1 family composed of Jun, Fos, and ATF. Interestingly, when interacting with Sp1/Sp3, these transcription factors can activate or repress transcription by recruiting cofactors inducing the opening or the closing of chromatin (Adams *et al*, 1995; Brodin *et al*, 2000; Hurst and Jones, 1987; Inoue *et al*, 1990; Kardassis *et al*, 1999; Laniel et al, 2001; Rafty *et al*, 2002; Roy and Guerin, 1994; Tapias *et al*, 2008). Consequently, hypermethylation in $GR1_B$ and 1_C promoters represses the binding of these

transcription factors reducing expression, but at the same time the hypomethylated state in $GR1_H$ permit Sp1/Sp3 binding and the recruitment of HDACs close the chromatin state. Such models are currently a matter of speculation, but serve to underscore the importance of studies of the molecular mechanisms that link methylation at specific genomic regions with alterations in transcriptional activity. These findings also point to the potential for bidirectional relation between transcription factor binding and transcriptional activity and that of DNA methylation (Berger, 2007; Meaney and Ferguson-Smith, 2010).

Another important consideration is that of interpreting data from studies of DNA methylation in the brain. DNA methylation is a digital signal; an allele is either methylated or unmethylated at a specific site in a given cell. The percentage of methylation measured in DNA methylation studies represents the fraction of cells in which the allele is methylated. An increase in methylation levels indicates an increase in the number of cells that bear a methylated allele. As expected for functional promoters, many of which lie within CpG islands, methylation levels are commonly low. GR promoters are generally hypomethylated in the majority of neurons in the hippocampus (Oberlander *et al*, 2008; McGowan *et al*, 2009; Alt *et al*, 2010; Turner *et al*, 2010), suggesting that these regions are poised for transcriptional activation in the majority of neurons. Our results suggest that site-specific methylation in selected GR promoters, such as exons 1_B, 1_C, 1_F, and 1_H, varies in a fraction of cells in the hippocampus as a function of childhood maltreatment. We suggest that this difference results in the downregulation of hippocampal GR expression.

Forebrain GR activation inhibits HPA activity through tonic negative-feedback inhibition (de Kloet *et al*, 2005). Thus, selective knockdown of GR expression in the corticolimbic system in rodents is associated with increased HPA activity under basal and stressful conditions (Barden, 2004; Boyle *et al*, 2005, Ridder *et al*, 2005). Conversely, GR overexpression is associated with a dampened HPA response to acute stress (Reichardt *et al*, 2000).

Familial dysfunction in childhood associates with increased CRF activity (Lee *et al*, 2005) and enhanced HPA and autonomic stress reactivity (DeBellis *et al*, 1994; Heim *et al*, 2000; Essex *et al*, 2002; Teicher *et al*, 2002; Luecken and Lemery, 2004; Pruessner *et al*, 2004). Importantly, interventions that target parental care of high-risk children alter HPA activity (Fisher *et al*, 2000). In humans, decreased GR expression, altered corticosteroid feedback sensitivity and increased HPA activity are linked to major depressive disorder (de Kloet *et al*, 2005; Neigh and Nemeroff, 2006). And there is evidence for decreased hippocampal GR expression in depression (Webster *et al*, 2002). Polymorphisms in the *NR3C1* gene that encodes the GR result in GR resistance and enhance the risk for major depressive disorder (van Rossum *et al*, 2006; van West *et al*, 2004). Although not all depressed patients show evidence for increased hypercortisolemia, psychotic and treatment-resistant forms of depression are commonly associated with increased HPA activity (Schatzberg *et al*, 1985; Holsboer, 2000). Interestingly, childhood maltreatment is associated with more severe, treatment-resistant forms of depression. Successful treatment of such populations with antidepressants may require a normalization of HPA activity (Holsboer, 2000). The GR antagonist, mifepristone (RU486), which blocks the effects of elevated cortisol, has been successfully used as an adjunct in the treatment of psychotic depression (DeBattista *et al*, 2006).

REVERSIBILITY OF DNA METHYLATION

The issue of reversibility is critical for translational studies of the epigenomic consequences of early adversity. To our knowledge, the issue has yet to be directly addressed in humans, even in samples of non-neural origin. Nevertheless there is considerable evidence that suggests a capacity for the remodeling of epigenetic marks over the lifespan, including DNA methylation. Across multiple tissues, including brain, the methylation levels at specific regions change with age (Hernandez *et al*, 2011), reflecting the potential for dynamic variation.

The results of the TSA study described above suggest that DNA methylation patterns are dynamic and potentially reversible even in adult animals. Infusion of the HDAC inhibitor resulted in a significant, partial reversal of the maternal effect on DNA methylation (Weaver *et al*, 2004). These findings are consistent with previous *in vitro* studies showing that increased histone acetylation associated with HDAC inhibitors can trigger demethylation (Szyf, 2009). Conversely, intra-hippocampal infusion of the methyl donor amino-acid methionine (Weaver *et al*, 2005) leads to a hypermethylation of the exon 1_7 GR promoter in the adult offspring of high LG animals. Thus, chronic central infusion of adult offspring of high or low LG mothers with methionine increases DNA methylation at the NGFI-A-binding site and reduces NGFI-A binding to the exon 1_7 promoter sequence selectively in the offspring of high LG mothers. These effects eliminate group differences in both hippocampal GR expression and HPA responses to stress. Methionine increases the levels of SAM and DNA methylation (Tremolizzo *et al*, 2005). SAM could increase DNA methylation through either the activation of DNA methylation enzymes (Pascale *et al*, 1991) or by inhibiting demethylase activity (Szyf *et al*, 2004). Likewise, studies of transcriptional regulation of *reelin* and *GAD1* reveal evidence for dynamic regulation of methylation states in mature cortical neurons through the disruption of repressor complexes and the inhibition of DNMT expression (Grayson *et al*, 2005; Kundakoic *et al*, 2007; Noh *et al*, 2005). Although the precise mechanisms for each of these effects is as yet unclear, these studies imply that mature brain cells express the enzymes necessary for both methylation and demethylation.

These findings are consistent with an emerging characterization of the potential for dynamic modifications in DNA methylation. Perhaps the most compelling evidence

for dynamic, experience-induced alterations in DNA methylation emerges from studies of contextual fear conditioning, a hippocampal-dependent learning paradigm whereby an animal associates a novel context with an aversive stimulus and is accompanied by broad increases in H3K9ac (Vecsey *et al*, 2007; Lubin *et al*, 2008; Miller and Sweatt, 2007; Sweatt, 2009), dependent upon activation of the ERK/MAPK signaling pathway and the CREB-binding protein. Dynamic changes in DNA methylation at specific genomic sites appear crucial for learning and memory (Day and Sweatt, 2011). Adult neurons show high levels of expression for the *de novo* methylation enzymes, DNMT3a and 3b. Moreover, there is considerable regional specificity in DNMT expression in the adult rat brain, suggesting a specialized function in adulthood (Brown *et al*, 2008). Increases in DNMT3a and 3b expression accompany contextual fear conditioning, and drugs that block DNMT activity impair conditioning (Miller and Sweatt, 2007). DNMT-deficient mice show impaired contextual fear conditioning (Feng *et al*, 2010). More recent studies identify specific genomic targets. Fear conditioning results in the methylation and transcriptional silencing of the gene for protein phosphatase 1, which associates with the suppression of learning (Sweatt, 2009). The same training results in the demethylation of a proximal promoter and transcriptional activation of the synaptic plasticity gene *reelin*.

These findings strongly imply that both DNA methylation and demethylation might be involved in the activity-dependent signaling pathways that underlie long-term memory consolidation. Brain-derived neurotrophic factor is an important example (Martinowich *et al*, 2003). More recent studies Guo *et al*, (2011) used next-generation sequencing for a genome-wide analysis of CpG methylation of adult mouse dentate granule neurons *in vivo* before and after synchronous neuronal activation (electroconvulsive stimulation; Guo *et al* (2011)). About 1.4% of the CpGs examined showed rapid active demethylation or *de novo* methylation, with some modifications remaining stable for at least 24 h. These activity-modified CpGs showed a broad genomic distribution with significant enrichment in low-CpG-density regions, and were associated with brain-specific genes related to neuronal plasticity. The low-CpG-density regions are of interest as the tightest correlations between DNA methylation and transcription are observed in such regions (Weber *et al*, 2007).

Taken together these findings suggest considerable capacity for active remodeling of DNA methylation. These findings are consistent with the prominent expression of DNMTs in neurons over adulthood and the degree to which DNMT expression as well as that of candidate demethylating agents is dynamically regulated by activity-dependent extracellular signals (Grayson *et al*, 2009; Ma *et al*, 2009). As treatments that target histone acetylation, such as HDAC inhibitors, can influence DNA methylation (Szyf, 2009), it might be possible to affect changes in DNA methylation through more accessible targets such as the histone post-translational modifications that directly regulate chromatin structure. However, the pathways that lead to the remodeling of DNA methylation, especially those implicated in DNA *de*methylation, have yet to be fully identified. A related question concerns the variability across in the genome in the capacity for epigenetic remodeling. We have yet to identify the factors that determine the sensitivity of genomic regions to active remodeling. One interesting possibility is that such variation, either across genomic regions or within the same genomic regions and across individuals, may be related to underlying sequence variation (Zhang *et al*, 2010). For example, methylation of a *BDNF* exon is associated with the well-known rs6265 (val66met) single-nucleotide polymorphism in the *BDNF* gene (Mill *et al*, 2006). Interestingly, the same polymorphism interacts with early-life adversity to influence hippocampal volume and the risk for depression (Gatt *et al*, 2009).

We have seen no underlying sequence variants in the various exon 1 regions of the *NR3C1* gene in the course of our sodium bisulfite mapping with either rat or human samples. However, such findings do not preclude variants at other sites, including those regions affecting the relevant intracellular signaling pathways. Studies linking genomic sequence variants to differential sensitivity to intervention (eg, Bakermans-Kranenburg *et al*, 2008) beg the question of whether such individual differences suggest a variation in the capacity for epigenetic remodeling.

SUMMARY

The results of the studies suggest that epigenetic mechanisms serve to mediate the association between early childhood and gene expression, and thus to explain, in part at least, individual differences in vulnerability/resistance for specific forms of psychopathology. We focused on the regulation of hippocampal GR expression as a model and provide evidence for parental effects on hippocampal GR expression that associate with differences in the methylation of exon 1 promoters. There is now evidence for comparable environmental effects at multiple regions of the genome (eg, Roth *et al*, 2009; Murgatroy *et al*, 2009).

Indeed the value of the energetically costly brain is to guide the function of the organism in accordance with its life history. The ability to mastermind such adaptation to circumstance relies upon the capacity of neurons and glia to dynamically adapt genomic structure and function (Meaney and Ferguson-Smith, 2010). The implicit hypothesis is that environmental signals alter chromatin modifications that then serve as the mechanism for the transcriptional 'plasticity' that mediates sustained variation in neural function. Ironically, the dynamic nature and environmental sensitivity of DNA methylation in fully differentiated cells is somewhat at odds with the very stability that suggests DNA methylation as a mechanism for parental effects on gene expression. How do we square the dynamic nature of DNA methylation with the phenotypic 'programing' associated with parental effects? We actually know rather little about the variation in methylation marks at specific loci over time

within the same individual. Perhaps a similar caveat applies to parental effects, studies of which often take the form of characterizing a parental signal at one stage of development, and then examining epigenetic states and phenotype at a later phase of life. The process of cell specialization that defines neural development depends upon the silencing on non-neural genes. This process can be activated *in vitro* in stem cells and the resulting repression initially involves Histone 3 lysine 27 tri-methylation, a polycomb-mediated, repressive histone modification (Mohn *et al*, 2008; and also see Cedar and Bergman, 2009). Repression then comes to reflect increased DNA methylation as neural differentiation proceeds, which is then thought to stabilize gene silencing. However, multiple regions of the genome in neural tissues are enriched for bidirectional histone modifications (ie, those associated with transcriptional activation, such as Histone 3 lysine 4 tri-methylation, as well as repression, notably H3K27me3), a characteristic of pluripotent cells (Bernstein *et al*, 2005). Such 'bi-valency' might define the potential for plasticity (Meaney and Ferguson-Smith, 2010). Repressive epigenetic contexts occur as a function of different epigenetic repertoires, which may vary in reversibility and confer variable environmental sensitivity (McEwen and Ferguson-Smith, 2010).

The challenge is that of defining causal pathways between environmental event, epigenetic mark, and genome function. In the context of the research on the parental regulation of hippocampal GR expression, future studies will need to focus on the mechanisms by which differential methylation of the exon 1 sequences affect transcription. A particularly interesting issue will be that of defining the processes that determine the relative stability and reversibility of parental effects.

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DISCLOSURE

The authors declare no conflict of interest.

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