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Methylation of exons 1_D, 1_F, and 1_H of the glucocorticoid receptor gene promoter and exposure to adversity in preschool-aged children

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Abstract

Epigenetic modifications to the genome are a key mechanism involved in the biological encoding of experience. Animal studies and a growing body of literature in humans have shown that early adversity is linked to methylation of the gene for the glucocorticoid receptor (GR), which is a key regulator of the hypothalamic–pituitary–adrenal axis as well as a broad range of physiological systems including metabolic and immune function. One hundred eighty-four families participated, including $n = 74$ with child welfare documentation of moderate-severe maltreatment in the past 6 months. Children ranged in age from 3 to 5 years, and were racially and ethnically diverse. Structured record review and interviews in the home were used to assess a history of maltreatment, other traumas, and contextual life stressors, and a composite variable assessed the number exposures to these adversities. Methylation of regions 1_D, 1_F, and 1_H of the GR gene was measured via sodium bisulfite pyrosequencing. The composite measure of adversity was positively correlated with methylation at exons 1_D and 1_F in the promoter of the GR gene. Individual stress measures were significantly associated with a several CpG sites in these regions. GR gene methylation may be a mechanism of the biobehavioral effects of adverse exposures in young children.

Developmental scientists have long understood that there is a complex and dynamic interplay between the person and the environment, and that experience shapes the development of affect and behavior. Recent decades have seen the emergence of a detailed understanding of the neurobiological systems that are influenced by the environment and are involved in the pathogenesis of psychiatric disorders. In the last several years, the mechanisms governing the translation of exposures to environmental influences into physiological change have begun to be clarified (Tyrka, Burgers, Philip, Price, & Carpenter, 2013). We now know that experience fundamentally alters the genome via epigenetic modifications that may be programmed early in development to alter neurobiological function.

Overview of Epigenetics

Epigenetic modifications to the genome alter gene expression but do not change the DNA sequence (Moore, Le, & Fan, 2012; Szyf, 2007). This allows for elaboration of the genome beyond what is determined by DNA base coding (i.e., genes). Methylation is the simplest, most stable, and most thoroughly examined form of epigenetic alteration (Szyf, 2007). Methylation involves the addition of a methyl group at sites in the DNA where a cytosine nucleotide occurs next to a guanine nucleotide (CpG dinucleotides). CpG sites are underrepresented in the genome, and they occur more commonly in gene promoter regions known as CpG islands. Methylation at CpG sites, particularly methylation at multiple cytosines within a CpG island, can result in transcriptional silencing of the gene so that it is not expressed. Mechanisms of gene silencing due to methylation include blocking of transcription factor binding and the recruitment of gene repression proteins to create and maintain a silenced chromatin conformation (Moore et al., 2012; Szyf, 2007).

Sensitive Periods, Neuroplasticity, and Glucocorticoid Signaling

The genetic code is the same in all DNA-containing cells in the body, yet cells vary dramatically in structure and activity depending on the organ system and function they serve. Very early in development, epigenetic processes program cell differentiation by regulating gene expression in a cell-type-specific fashion. In addition to these fundamental effects on cell differentiation during embryogenesis and fetal development,

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epigenetic modifications regulate many genes that are involved in coordinating a broad range of biological processes (Moore et al., 2012; Szyf, 2007). Such changes can occur in response to environmental exposures during fetal development and may also occur in childhood (Zhang, Labonte, Wen, Turecki, & Meaney, 2013). The machinery for methylation and demethylation processes is present even into adulthood (Moore et al., 2012); however, the extent to which these programs can be altered later in life is not known (Zhang et al., 2013).

Much of the work on epigenetics in relation to early experiences has focused on the glucocorticoid receptor (GR) gene (nuclear receptor subfamily 3, group C, member 1 [*NR3C1*]). GRs are widespread throughout the limbic system as well as the immune and other systems where they regulate the coordinated physiological response to threats. In addition to effects of glucocorticoids (cortisol in humans) on target tissues, the GR mediates negative-feedback regulation of the hypothalamic–pituitary–adrenal (HPA) axis. Converging evidence from animal models and human studies implicate impairments in glucocorticoid signaling in major depressive disorder and posttraumatic stress disorder (PTSD), including alterations of GR number and function in the brain and in peripheral cells such as leukocytes (Barden, 2004; Provencal et al., 2012; van Zuiden et al., 2011; Yehuda et al., 2010; Yehuda & Seckl, 2011). Although access to brain tissue is limited to animal and postmortem human studies, given the widespread and coordinated role that glucocorticoids play in the stress response, some coregulation of peripheral and central GRs is likely (Szyf, 2012, 2013).

The hippocampus and amygdala are particularly important in the response to stress and regulation of affect and behavior. There is a high density of GRs in the hippocampus and amygdala, and animal models show that exposure to stress or glucocorticoids, along with effects on other stress mediators, alters neural structure in these regions. In the hippocampus, chronic stress and glucocorticoid treatment inhibit neurogenesis, cell proliferation, and dendritic branching, and induce cell loss and atrophy (Duman, 2009). In contrast, in the amygdala, which mediates fear responses, chronic stress may induce a proliferative effect on neuronal dendritic branching and spine density (Vyas, Jadhav, & Chattarji, 2006). Findings from animal models and human neuroimaging studies indicate that these limbic brain structures are highly vulnerable to the effects of early-life stress and trauma (Lupien, McEwen, Gunnar, & Heim, 2009; Tottenham & Sheridan, 2009). These data are complex, but converging evidence indicates that the timing of stress exposure in relation to the time frame for development of these structures is critical (Lupien et al., 2009; Tottenham & Sheridan, 2009).

Rodent Models of Early Environment and GR Methylation

Work in rodents examining naturally occurring differences in maternal care has provided compelling evidence of effects of environmental stimuli on epigenetic alteration of the GR gene in the hippocampus (Zhang et al., 2013). Rat pups reared by dams with low frequency of maternal behaviors (licking/grooming and arched-back nursing) had higher levels of methylation of the rat hippocampal GR gene promoter (exon 1_7 of the promoter region of the rodent GR gene, *nr3c1*). The differentially methylated region included a binding site for the transcription factor, nerve growth factor inducible factor-A (NGFI-A). Consistent with a gene-silencing effect of methylation, methylation was associated with reduced *nr3c1* gene expression and decreased numbers of GRs in the hippocampus. Moreover, these changes were associated with exaggerated glucocorticoid secretion and behavioral signs of distress (Weaver et al., 2004; Zhang et al., 2013). Because the GR plays an important role in modulating HPA axis responses, reduced numbers of GRs may limit the regulatory capacity of this system.

In addition to naturally occurring maternal behaviors, a number of well-validated models allow experimenters to introduce early stressors that disrupt caregiving behaviors. Numerous studies have found effects of early stress models on methylation of *nr3c1* and other genes that regulate HPA axis function (Gudsnuk & Champagne, 2014; Kember et al., 2012; Kundakovic, Lim, Gudsnuk, & Champagne, 2013), but not all findings have been positive for methylation of *nr3c1* (Daniels et al., 2009; Lee et al., 2010) and some findings were specific to strain or sex differences (Kember et al., 2012; Kundakovic et al., 2013). These findings suggest variability and moderators of the effects of environment on *nr3c1* methylation and expression in rodent models of stress exposure; in humans, of course, the situation is likely to be far more complex.

Human Development: Contextual Stressors

Childhood adversity is a two-generation issue embedded in community context. The individual and contextual circumstances of parents are transmitted directly and indirectly to the child generation. This begins during pregnancy, where factors such as adolescent pregnancy, unattended maternal health problems, poor nutrition, stressful experiences, and preterm birth confer risks to newborn children (Ventura, Hamilton, & Mathews, 2013). As children develop, they begin to experience directly the stressful environments into which they were born. Like their parents, children may be exposed to environmental toxins, may receive poor health care, and may be poorly nourished. The behaviors of adults in their environments disproportionally result in psychosocial stress and environmental toxins experienced by these children. Contextual stressors at each level of the environment often co-occur, and children in poverty are commonly exposed to multiple co-occurring risks (Evans, Gonnella, Marcynyszyn, Gentile, & Salpekar, 2005; Sameroff, Seifer, Baldwin, & Baldwin, 1993).

Associations of Early Stress and Human GR Gene Methylation

Despite this complexity, several studies have now shown that particular indicators of childhood stress are linked to methylation

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of the exon 1_F region of the human gene (*NR3C1*) promoter, which is homologous to the rat exon 1_7 and contains an NGFI-A binding site. Oberlander et al. (2008) were the first to examine this issue in humans. A region of this exon was examined in cord blood DNA from 82 infants. Higher levels of methylation at three CpG sites were associated with prenatal exposure to maternal depressive and anxiety symptoms. One of these regions is a binding site for NGFI-A, and methylation at this site was positively associated with salivary cortisol responses to infant stimulation at 3 months. McGowan et al. (2009) examined an adjacent, but not overlapping, region of exon 1_F (Figure 1) in postmortem hippocampus tissue from 36 men: 12 suicides with proxy-based interview reports of childhood abuse, 12 suicide victims without known abuse, and 12 men who died from other causes. The suicide-abuse group had increased methylation at a number of CpG sites, methylation was linked to decreased binding of NGFI-A, and there were decreased levels of GR mRNA compared to the other groups. This was the first, and only existing study thus far, to examine *NR3C1* methylation in brain tissue in relation to early experience, and the findings provide strong evidence that early experience may be translated into neural effects through this mechanism.

Prenatal Exposure to Maternal Symptoms and Stress

A number of recent studies have confirmed effects of maternal antenatal depression, anxiety, or stress exposure on methylation of *NR3C1* in placenta or cord blood. The placenta is a key regulator of intrauterine development, and there is evidence that placenta *NR3C1* methylation confers impairments in infant attentional and motor processes (Bromer, Marsit, Armstrong, Padbury, & Lester, 2012). In a sample of 482 mother–infant pairs, Conradt, Lester, Appleton, Armstrong, and Marsit (2013) studied the region of exon 1_F examined by Oberlander et al. Prenatal exposure to maternal depression was linked to greater placenta methylation of *NR3C1*, as well as infant hypotonia, lethargy, and poor self-regulation. Hompes et al. (2013) examined psychological symptoms in a sample of 83 predominantly Belgian women and found that anxiety related to pregnancy was associated with higher methylation at exon 1_F in cord blood.

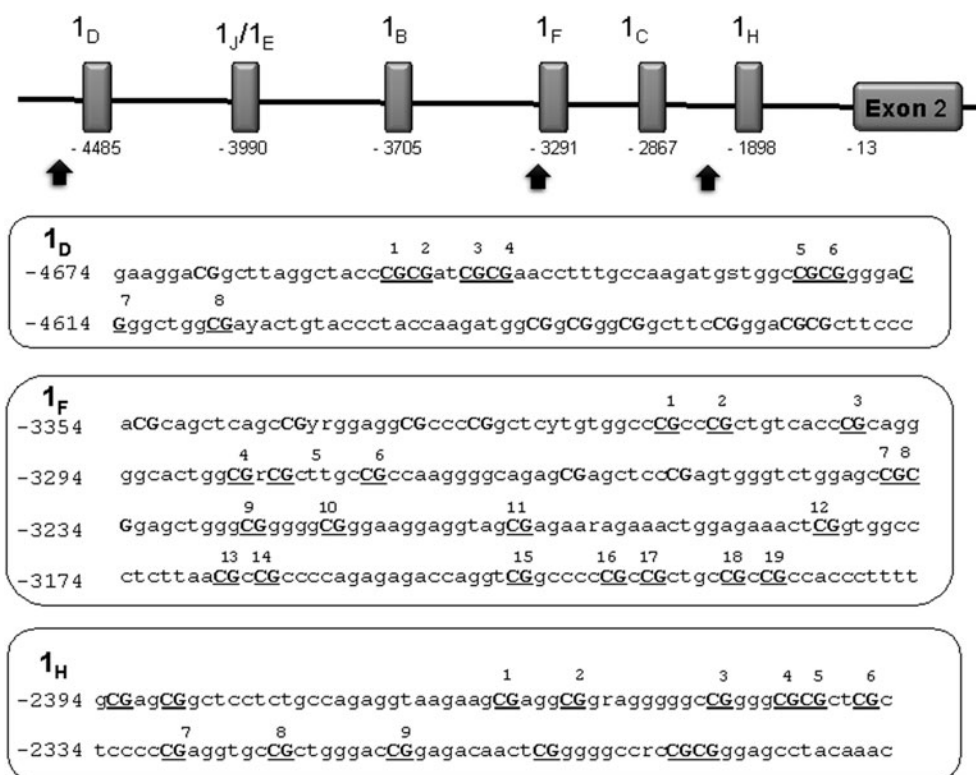

Figure 1. Schematic diagram of noncoding exons in the glucocorticoid receptor gene promoter region. (Top) The number below each noncoding exon represents the distance to the translational start located at –13 nucleotides upstream of the start of exon 2. The arrows represent the regions sequenced in this study, the promoter of exon 1_D, promoter and exon 1_F, and promoter of exon 1_H. (Bottom) The boxes contain the genomic sequences analyzed for each region. The analyzed CpG loci are underlined and numbered according to each exon region. Promoter 1_D: Investigated by Hompes et al. (2013), psychological state components and maternal cortisol in T2 predicted 18.6% of the variance of the epigenetic state of CpG-unit D6.8 in cord blood (note unit D6.8 corresponds to CpG 4, 5, and 6 in Assay 1_D). Promoter and exon 1_F: CpG 1–6 corresponds to CpG 27–32 in the sequence in McGowan et al. (2009), CpG 7–19 corresponds to the region studied by Oberlander et al. (2008). Promoter 1_H: CpG1–9. This region was found by Labonte et al. (2012) to be hypomethylated in the hippocampus, but not the anterior cingulate of suicide victims with history of childhood abuse.

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In addition to findings for maternal depressed mood and anxiety, there is evidence that maternal stress and trauma exposure is linked to neonatal *NR3C1* methylation. Mulligan, D'Errico, Stees, and Hughes (2012) investigated a sample of 25 mother–newborn pairs in July–August 2010 from eastern Democratic Republic of Congo, a war-torn region with extreme violence toward women, and found that maternal exposure to war stress (including rape, refugee status, and other stressors) was linked to greater cord blood methylation of the region studied by McGowan et al. In a retrospective study of 25 youth age 10–19 and their mothers, Radtke et al. (2011) found that methylation of this same region of exon 1_F in leukocytes in youth was associated with maternal reports of intimate partner violence during pregnancy, but not intimate partner violence that occurred before or after pregnancy. This finding suggests the possibility that epigenetic effects that occur in utero may have long-lasting effects.

Adults With a History of Childhood Adversity

Several studies have also examined *NR3C1* promoter methylation in leukocytes of adults who reported on their history of early life experiences. Our group examined leukocyte DNA of 99 adults with no lifetime psychopathology in order to determine effects of adversity that are independent of effects of the disorders themselves. Greater methylation at the exon 1_F region first examined by Oberlander et al. was seen in subjects with childhood parental loss or a history of maltreatment, but was not associated with recent perceived stress (Tyrka, Price, Marsit, Walters, & Carpenter, 2012). In addition, increased methylation was significantly associated with an attenuated cortisol response to the dexamethasone/corticotropin-releasing hormone test. These findings suggest that altered *NR3C1* methylation may be a long-lasting mark of exposure even in the absence of psychopathology.

The remaining work in adults has examined exposure to childhood stress or trauma among patients with a psychiatric disorder. Although much of this work is confined to psychiatric patients, many of whom were taking psychotropic medications that may alter methylation patterns, there is evidence of an effect of reported childhood adversity. A study of adult patients with borderline personality disorder, major depressive disorder, or PTSD found that methylation of the Oberlander et al. region leukocyte exon 1_F *NR3C1* promoter methylation was positively associated with a history of childhood sexual abuse and with the severity of maltreatment (Perroud et al., 2011). In contrast, Steiger, Labonte, Groleau, Turecki, and Israel (2013) did not find an effect of childhood maltreatment on *NR3C1* 1_F promoter methylation in a sample of adult patients with bulimia nervosa. Melas et al. (2013) studied 285 women with a depressive disorder and 708 healthy women and found that childhood exposure to parental death was linked to higher saliva *NR3C1* exon 1_F methylation across all subjects. This was the first study to examine saliva methylation; the findings were consistent with those of studies measuring methylation of this exon 1_F in leukocytes and the brain.

Alternate First Exons

In addition to *NR3C1* exon 1_F, six alternate first exons are located in a CpG island upstream of exon 2, which encodes the translational start codon for the gene. These alternate first exons are differentially expressed according to tissue type and are thought to be involved in tissue-specific control of GR expression (Moore et al., 2012). These exons each contain binding sites for various transcription factors that can be regulated themselves in a tissue-specific fashion (Turner & Muller, 2005). Only a few studies have examined methylation of other *NR3C1* first exons in relation to childhood adversity. In another report from the study of postmortem hippocampal tissue, greater methylation of exons 1_B, and 1_C, but less methylation at 1_H, was found in the suicide-abuse group (Labonte et al., 2012). Steiger et al. (2013) found that in comparison with controls, patients with both bulimia and borderline personality disorder had significantly greater methylation at exon 1C and lower levels of methylation at exon 1_H; however, there were no effects of childhood maltreatment. Finally, Hompes et al. (2013) found positive effects of maternal symptoms on cord blood methylation in the *NR3C1* exon 1_D, and methylation in this region was linked to diurnal cortisol levels.

Limitations of Extant Literature and the Present Study

In summary, there have been a handful of studies investigating associations of maternal symptoms or maternal exposure to trauma on placenta or cord blood *NR3C1* methylation, one investigation of leukocyte *NR3C1* methylation in healthy adults with a history of childhood adversity, and a few studies of reported childhood maltreatment or parental loss in subjects with mood disorders or suicide. Investigations of maternal symptoms or of subjects with psychopathology could reflect processes specific to the disorder(s) rather than exposures per se. Retrospective reports from adults with a history of childhood adversity do not allow examination of the developmental timing of epigenetic effects of stress exposure, so knowledge of the developmental window during which stress can influence *NR3C1* methylation is limited. Retrospective reports of maltreatment may also introduce error in the ascertainment of the key contextual stressor under investigation. Finally, other than exon 1_F, very few studies have examined alternate first exons of *NR3C1*, and we are aware of only one study focused on *NR3C1* methylation in saliva DNA. In the present study, we examined methylation of three alternate first exons in the promoter of *NR3C1* in saliva DNA in a sample of socioeconomically impoverished preschool-aged children with and without recent maltreatment.

Methods

One hundred and eighty-four families enrolled in this study. One child from each family was included in the study. Children ranged in age from 3 to 5 years ($M = 50.3$ months; $SD =$

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8.5 months), were racially and ethnically diverse (41 White non-Hispanic, 87 Hispanic, 29 Black, and 27 other races), and 90 were male. Most caregivers ($n = 174$) were biological mothers. Thirty-three caregivers had less than a high school degree, 72 completed high school, 61 had some postsecondary education, 17 had a bachelor's degree, and 1 did not provide education information. One hundred and four caregivers were unemployed and 166 of the families qualified for public assistance. Seventy-four children (40.2%) had substantiated cases of moderate to severe child maltreatment within the past 6 months as described below.

Procedure

Families with a maltreated child were identified from the local child welfare agency and an emergency maltreatment assessment service via record review. Families of children with no indicated case of maltreatment within the past 6 months were recruited at a pediatric medical clinic during a well-child visit as well as at childcare centers. Based on review of available medical records and parent report, children with a chronic illness, medication use, obesity, and failure to thrive were excluded. Those with acute illness or medication use were included no less than 2 weeks following resolution of illness and medication use.

Families completed two home visits and questionnaires between the visits. The first home visit, during which caregivers completed interviews on child stress exposure and a saliva sample for DNA isolation was collected from the children, is the focus of the current report.

Measures

Socioeconomic adversity. Indicators of socioeconomic adversity (parental education ≤ high school degree, parental unemployment, and single parenthood) were summed.

Child maltreatment status. All families consented to examination of child welfare records to determine maltreatment status. Trained research staff coded the records using the System for Coding Subtype and Severity of Maltreatment in Child Protective Records (Barnett, Manly, & Cicchetti, 1993). Five maltreatment subtypes and severity scores ranging from 1 (*least severe*) to 5 (*most severe*) were derived. Children with a case of moderate to severe levels of maltreatment (score = 3–5) within the last 6 months were considered as part of the maltreated group ($n = 74$). Nine children had substantiated cases of physical abuse, 16 sexual abuse, 9 physical neglect/failure to provide, 20 physical neglect/lack of supervision, and 49 emotional maltreatment. Three of the maltreated children were removed from the home and were in the care of their maternal grandmother. The comparison group included children who had never had a substantiated case of maltreatment. In addition, 5 children had an episode of moderate maltreatment that occurred at least 18 months prior to participation. Results were consistent whether these children were in the maltreatment or the comparison group. Because 5 children is insufficient for a separate analysis, these children were included in the comparison group.

Contextual stress interview. Caregivers completed a semistructured interview developed in our laboratory to assess the child's experience of contextual stressors in the past month and in the child's lifetime. Categories were as follows: death of a caregiver, separation from a caregiver, frequent change of residence or homelessness, inadequate food or clothing, and other events, including witnessing neighborhood violence or parental arrest. Each domain was scored positive if at least one episode occurred, and domains were summed for past month and lifetime. Possible scores ranged from 0 (*no stressors*) to 5 (*stressors in all five domains*) for each summary scale. Past-month stressors ranged from 0 to 3 (mean = 0.64, $SD = 0.85$), and lifetime stressors ranged from 0 to 4 (mean = 1.39, $SD = 1.24$).

Traumatic life events. The Diagnostic Infant and Preschool Assessment (Scheeringa & Haslett, 2010) interview was conducted with caregivers to assess child experiences of traumatic life events. Interviews were conducted by trained clinical social workers and PhD-level psychologists, reviewed in a group supervision format, and scored based upon group consensus. Traumatic events in each domain were dichotomized (no trauma vs. ≥1 trauma), then summed to create a scale for number of types of traumas experienced in the child's lifetime. Physical and sexual abuse were not included because they were assessed as maltreatment (above). Possible scores ranged from 0 to 8, and in the present sample ranged from 0 to 4 (mean = 0.92, $SD = 1.02$).

Adversity composite. The number of types of maltreatment experienced, the number of lifetime contextual stressors, and the number of traumatic life events were summed to create an adversity composite. Possible scores ranged from 0 to 18, and in the sample ranged from 0 to 9 (mean = 2.92, $SD = 2.45$).

GR methylation

Saliva samples were obtained using the Oragene.DISCOVER kits (OGR-575) for Assisted Collections (DNA Genotek, Kanata, ON, Canada), and DNA was isolated following the manufacturer's instructions. Sodium bisulfite modification was performed with 500 ng of DNA using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). For DNA methylation detection, bisulfite pyrosequencing was employed in three locations within the *NR3C1* region: promoter of exon 1_D, promoter and exon 1_F (three assays), and promoter of exon 1_H (Figure 1). PyroMark Assay Design software version 2.0.1.15 (Qiagen) was used to design the pyrosequencing assays. Polymerase chain reaction, sequencing primers (Integrated DNA Technologies, Inc., Coralville, IA), and pyrosequencing assay sequences are available from the corresponding author upon request. The PyroMark polymerase

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chain reaction kit and forward and reverse primers were used to amplify specific regions of the *NR3C1* promoter. Amplification cycling conditions were as follows: 94 °C for 15 min followed by 45 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min with a final extension of 10 min at 72 °C. Five forward pyrosequencing assays covering a total of 36 CpG loci were performed in triplicate using the PyroMark MD (Qiagen). Non-CpG cytosines within assays served as internal controls to verify bisulfite DNA modification efficiency. Each run included a nontemplate control. Percent DNA methylation at each CpG locus was quantified with the PyroMark CpG software, version 1.0.11 (Qiagen). All procedures were performed following manufacturer's protocols.

The percentage of alleles that were methylated was used in statistical analyses. Consistent with other studies on *NR3C1* methylation in a variety of cell types, methylation levels were low across these regions. For CpG sites in region 1_D, the percentage of methylation ranged from 0 to 7.02 (mean = 1.10, *SD* = 0.19) across the whole region. Region 1_F methylation varied from 0 to 6.39. The mean and *SD* of the whole region were 1.45 and 0.26, respectively. For 1_H, the range of methylation was 0 to 6.92, and the mean across the region was 1.23 (*SD* = 0.22).

Results

Preliminary analyses

Child age, race, and socioeconomic adversity were not associated with mean methylation at any of the *NR3C1* first exons. Males had greater mean methylation at 1_F than females (p = .013), so sex was included as a covariate when testing associations of adversity variables with 1_F. Intercorrelations of mean methylation levels at exons 1_D, 1_F, and 1_H were all below 0.30.

Associations of adversity variables with NR3C1 methylation

Region 1D. Table 1 displays associations of the adversity variables on mean methylation at region 1D and the other first exons. The adversity composite was positively associated with mean methylation across exon 1_D. Child maltreatment was also individually associated with greater methylation of exon 1_D. Examination of the individual CpG sites revealed significant effects at CpG 5 for the adversity composite ($r = .17$, $p = .024$) and maltreatment, t (182) = 3.14, p = .002. There were also trend-level effects of the composite adversity variable at CpG 3 ($r = .13$, $p = .083$) and CpG 4 ($r = .13$, $p = .069$). Neither the number of traumas nor the contextual stress measures was associated with the mean across 1D sites; however, exploration of individual CpGs revealed that lifetime stressors were associated with greater methylation at CpG 4 ($r = .16$, $p = .034$) and CpG 5 ($r = .18$, $p = .017$), and past-month stressors showed trend-level associations with CpG 4 ($r = .12$, $p = .095$) and CpG 5 ($r = .13$, $p = .079$).

Table 1. *Associations of adversity variables with mean methylation at alternate first exons*

	1_D	1_F	1_H
Adversity composite	$r = .15$*	$r = .16$*	$r = -.01$
Child maltreatment status	$t = 2.62$**	$F = 0.66$	$t = 0.10$
Past month contextual stress	$r = .08$	$r = .20$**	$r = -.07$
Lifetime contextual stress	$r = .14$	$r = .19$**	$r = -.07$
Number of traumas	$r = .06$	$r = .07$	$r = -.01$

Note: Child sex was included as a covariate when testing associations of adversity variables with 1_F. The adversity composite was the sum of the number of types of maltreatment, number of lifetime contextual stressors, and number of traumas.
*$p < .05$. **$p < .01$.

Region 1F. The adversity composite was also positively associated with mean methylation at exon 1_F (Table 1). The number of contextual stressors in the past month and the number of lifetime contextual stressors were also significantly correlated with mean methylation at 1_F (Table 1). Examination of the individual CpG sites revealed significant effects of composite adversity and lifetime stressors on methylation at CpG 3 ($r = .23$, $p = .002$ and $r = .21$, $p = .004$, respectively) and CpG 4 ($r = .20$, $p = .008$ and $r = .20$, $p = .007$, respectively), and a trend for lifetime stressors and methylation at CpG 17 ($r = .14$, $p = .062$). Stress exposure in the past month was positively correlated with methylation at CpG 2 ($r = .19$, $p = .011$), CpG 3 ($r = .17$, $p = .022$), CpG 14 ($r = .15$, $p = .038$), CpG 17 ($r = .17$, $p = .021$), and CpG 19 ($r = .15$, $p = .046$). There was no effect of child maltreatment or the number of traumatic events on mean methylation at region 1_F (Table 1). However, exploratory analysis of individual CpG sites showed that maltreatment was associated with greater methylation at CpG 3, F (1) = 6.70, $p = .010$, and the number of traumatic events was associated with greater methylation at CpG 4 ($r = .18$, $p = .019$) and at trend level for CpG 3 ($r = .13$, $p = .086$).

Region 1H. There were no associations of any of the adversity variables with mean methylation at exon 1_H (Table 1). Exploratory analysis of methylation at the individual CpG sites also did not reveal any associations with adversity measures at 1_H.

Discussion

In this study, we found that stress exposure during the preschool period is linked to higher levels of *NR3C1* promoter methylation in exon 1_F and exon 1_D in DNA obtained from saliva. To our knowledge, this is the first published study of stress exposure and methylation of *NR3C1* promoter sites in young children, the first to study children with documented maltreatment, and one of the first to study effects of stress

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on *NR3C1* promoter methylation in saliva or buccal cell DNA. While effect sizes are small to moderate, they are consistent with prior studies of *NR3C1* showing effects of prenatal exposures on promoter methylation in cord blood and placenta, studies of leukocyte methylation in healthy adults with early stress and trauma and in youth with a history of perinatal stress exposure, and an investigation of promoter methylation in hippocampus tissue from postmortem brains. Our results extend findings from prior work to show that (a) exposure to stress in the preschool period is linked to *NR3C1* methylation, (b) differential methylation of *NR3C1* is seen in DNA from saliva, (c) in addition to exon 1_F, exon 1_D is involved, and (d) contextual stressors are significant determinants of *NR3C1* methylation in an impoverished sample of children with and without identified maltreatment.

We examined the association of childhood stress exposure with *NR3C1* promoter methylation in saliva DNA. There is evidence that, unlike buccal cell techniques, saliva DNA may originate from blood leukocytes (Endler, Greinix, Winkler, Mitterbauer, & Mannhalter, 1999; Thiede, Prange-Krex, Freiberg-Richter, Bornhauser, & Ehninger, 2000). However, because our young children were not able to provide saliva via passive drool, we used a standardized method that involves use a sponge to collects pooled saliva beneath the tongue and at the intersection of the cheek and gum, so that in addition to leukocytes, it is likely to yield some epithelial cells. Essex et al. (2013) recently published the only study of childhood stress exposure and buccal epithelial cell methylation and found that multiple genes were differentially methylated in buccal-cell DNA. While there was no effect for *NR3C1*, only one CpG site from this gene was included in their whole-genome array. Melas et al. (2013) examined *NR3C1* promoter methylation in saliva and found effects consistent with our findings and with prior investigations in leukocytes and placental and hippocampus tissue. However, we cannot rule out the possibility that our findings might be influenced by an association of stress with the proportion of epithelial and leukocytes present in the sample. Altered distribution of leukocyte cell type may be an issue in studies of leukocyte methylation; however, Talens et al. (2010) found that leukocyte cell type was not associated with variation in *NR3C1* methylation.

Consistent with other studies of methylation in these alternate first exons of *NR3C1*, we found quite low overall levels of methylation. It is not clear whether such low levels of methylation would substantially alter gene expression; however, McGowan et al. (2009) found that *NR3C1* promoter methylation was associated with decreased gene expression in hippocampus tissue, and there is evidence that leukocyte and cord blood methylation are associated with diurnal or provoked cortisol concentrations (de Rooij et al., 2011; Edelman et al., 2012; Hompes et al., 2013; Tyrka et al., 2012). There are numerous known or putative transcription factor binding sites throughout the regions we examined (Akiyama, 1995; Armstrong, Lesseur, Conradt, Lester, & Marsit, 2014; Heinemeyer et al., 1998). In addition, it is not known whether peripheral regulation of GR number and function parallels methylation of brain regions involved in the regulation of mood, cognition, and behavior, though findings of similar associations of early stress with *NR3C1* methylation in peripheral cells and hippocampus tissue provide evidence of common effects. Moreover, given the effects of early stress and glucocorticoid signaling on a wide range of systems, including metabolism and immune function, peripheral tissues are of interest in their own right.

Our findings for *NR3C1* promoter exon 1_F extend the literature on methylation of this exon and in utero exposures and reported childhood maltreatment in adults with psychopathology and suicides. Most of our positive findings for this region were concentrated in the area first examined by McGowan et al., where methylation of this region of *NR3C1* in the hippocampus was higher in suicides with a history of childhood abuse. Our region identified as CpG 3 and 4 corresponded to McGowan sites 29 and 30; their significant effects for abuse in the context of suicide were at CpG 30 and 32. That maltreatment and other adverse experiences were associated with greater methylation at exon 1_D adds to findings of Hompes et al. (2013) of higher methylation of 1_D in leukocytes in association with maternal psychological symptoms and cortisol. The CpG sites that were methylated in association with stress and trauma in our study corresponded to the region that was predicted by cortisol levels in multivariate analyses controlling for maternal symptoms in the study by Hompes et al. (2013).

That childhood maltreatment per se did not have larger or more widespread effects on *NR3C1* promoter methylation in this sample may be because the sample as a whole was socioeconomically impoverished, with contextual stress exposure in nonmaltreated and maltreated subjects alike. In addition, it is possible that some of the children without a child protective services record of maltreatment nevertheless had experienced abuse or neglect. That we showed effects of measured stress exposure at all suggests that larger effects would be seen with a nonstressed, high socioeconomic status comparison group.

We did not find associations of stress exposure with methylation of exon 1_H. This is in contrast to findings from the prior studies of maltreatment and postmortem hippocampus tissue (Labonte et al., 2012) and psychopathology and leukocyte DNA (Steiger et al., 2013). However, Labonte et al. (2012) found no associations of childhood maltreatment with any of the examined *NR3C1* regions in the anterior cingulate, suggesting tissue-specific differences in the association of stress with methylation of this alternate first exon. Further studies are needed to clarify the role of methylation at this exon in relation to stress exposure and psychopathology.

Given the prenatal programming effects of methylation in general and the prior work showing effects of exposure to maternal symptoms and stressors, it is possible that some of our effect is due to prenatal exposures. However, while there may be some association between contextual stressors during the preschool period and prenatal exposures, these contextual

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stressors are common in poverty and are unlikely to be determined by earlier stressors such as intimate partner violence. We also did not measure maternal methylation or psychopathology, which could be contributory. However, there is evidence in humans that maternal and offspring methylation at these sites are not significantly correlated (Mulligan et al., 2012; Oberlander et al., 2008; Radtke et al., 2011), suggesting that these methylation marks are not heritable across generations and are driven by environmental regulation during the early developmental period. Cross fostering studies also show that the effects of maternal behaviors on *nr3c1* methylation in rodents are due to effects of exposures, not inheritance (Zhang et al., 2013). However, exposure-induced methylation of some genes can be directly inherited through the germline (Dias & Ressler, 2014; Gudsnuk & Champagne, 2014).

A number of studies have found differential methylation of several other genes, including those that regulate GR function and other components of the biological response to stress (Lutz & Turecki, 2013), and recent work demonstrates moderating influences of some of these genes on *NR3C1* methylation in relation to stress exposure (Klengel et al., 2013; Melas et al., 2013). Recent genome-wide studies of childhood adversity and/or PTSD have identified differentially methylated genes across the genome (Essex et al., 2013; Lutz & Turecki, 2013; Smith et al., 2011). It should be noted, however, that large arrays, which examine hundreds of thousands of genes, may not identify individual genes of interest due to reduced sensitivity or reduced coverage of CpG sites in each gene. In this study, we employed bisulfite pyrosequencing, a highly sensitive method that allows detection of low levels and mixed levels of DNA methylation (Reed, Poulin, Yan, & Parissenti, 2010). Dammann et al. (2011) found that *NR3C1* and other genes linked to psychopathology were differentially methylated in patients with borderline personality disorder only when examined via pyrosequencing, not when using bisulfite restriction analysis, which would provide more limited coverage based on location of restriction sites.

Given converging evidence that the biological encoding of environmental exposures occurs through epigenetic modification of genes that regulate stress responding, affect, and behavior, a major question for future research is how to target these mechanisms using intervention and prevention approaches. Methylation was previously thought to be fixed during early development, when it serves to set up trajectories that program further development. Few studies have been conducted in humans, but emerging research suggests the possibility that some epigenetic processes may be dynamic over the life span, might be reversed in response to interventions, or might predict treatment response (Champagne & Curley, 2009; Gudsnuk & Champagne, 2014; Melas et al., 2013; Tadic et al., 2014; Yehuda et al., 2013). Given the critical role of epigenetic modifications in programming cell differentiation and cellular function, the determinants and limitations of such dynamic lability in methylation status remain to be delineated and will require a great deal more study.

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