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HHS Public Access

Author manuscript

Dev Psychopathol. Author manuscript; available in PMC 2018 December 01.

Published in final edited form as:

Dev Psychopathol. 2017 December ; 29(5): 1795–1806. doi:10.1017/S0954579417001407.

Methylation of the glucocorticoid receptor gene (*NR3C1*) in maltreated and nonmaltreated children: Associations with behavioral undercontrol, emotional lability/negativity, and externalizing and internalizing symptoms

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Abstract

The present study examined the effect of various dimensions of child maltreatment (i.e., developmental timing of maltreatment, number of maltreatment subtypes, and chronicity of maltreatment) on methylation of the glucocorticoid receptor (GR) gene, NR3C1, and investigated the associations between NR3C1 methylation and child outcomes. Participants included 534 children who attended a research summer camp program for school-aged maltreated (53.4%) and nonmaltreated (46.6%) children from low socioeconomic backgrounds. Results show that children with early-onset maltreatment evidence significant hypermethylation compared to nonmaltreated children. Moreover, more maltreatment subtypes experienced and more chronic maltreatment are both related to greater NR3C1 hypermethylation. Findings also indicate that hypermethylation of NR3C1 is linked with a number of negative child outcomes including greater emotional lability-negativity, higher levels of ego undercontrol, more externalizing behavior, and greater depressive symptoms. Together, results highlight the role of methylation of NR3C1 in the effects of child maltreatment on the development of emotion dysregulation and psychopathology.

Keywords

epigenetics; child maltreatment; dimensions of child maltreatment; NR3C1; DNA methylation

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Early caregiving experiences have been shown to play a critical role in shaping brain and behavioral development and physical health (Cicchetti, Hetzel, Rogosch, Handley, & Toth, 2016a, b; DeBellis, 2001; Doyle & Cicchetti, 2017; Hertzman & Boyce, 2010; Rutter, 2012, 2016; Weaver, Cervoni, D'Aleasio, Champagne, Seckl et al., 2004). Thus, children whose early experiences are marred by absent or abusive caregiving are denied opportunities for healthy development (Cicchetti & Lynch, 1995; Cicchetti & Toth, 2016; Tyrka, Price, Marsit, Walters & Carpenter, 2012; Tyrka, Ridout, & Parade, 2016). Instead, adverse caregiving environments can usher in motion probabilistic developmental pathways that are

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characterized by an increased risk for atypical brain development, relationship difficulties, maladaptive behavior, and psychopathology across the life span (Cicchetti, 2002; Cicchetti & Toth, 2016; Essex et al., 2013; Szyf & Bick, 2013; Turecki & Meaney, 2016; Zhang & Meaney, 2010). Child maltreatment sensitizes neural function and neuroendocrine responses to stress exposure, thereby bringing about a vulnerability to psychopathology, such as depression and internalizing and externalizing problems (Caspi, et al., 2002, 2003; Hart, Gunnar, & Cicchetti, 1996; Kaplow & Widom, 2007; Thibodeau, Cicchetti, & Rogosch, 2015; Toth, Manly & Cicchetti, 1992; Turecki & Meaney, 2016).

In order to understand the processes through which early adversity imparts maladaptive development and/or psychopathology, it is essential that both genotypic variation and epigenetic alterations are examined. Early childhood caregiving environments work together with genotypic variation and epigenetic regulation to affect biological and psychological development throughout the life course (Hertzman, 2012). Epigenetics has been conceived as a potential mechanism for how adversity in early life confers risk for lifelong biological and psychological problems (Lester, Conradt, & Marsit, 2016; Szyf & Bick, 2013; Tyrka et al., 2016). Epigenetics involves functionally relevant changes to the genome that do not eventuate in alterations in the nucleotide sequence. Epigenetic modifications can impart changes in gene expression and neural function without bringing about alterations in the underlying DNA sequence (Zhang & Meaney, 2010). Research with rhesus macaque monkeys raised maternally support the hypothesis that the response to maternal care is not limited to one tissue or one brain region, but indicated that the impact of early life adversity is system-wide and genome-wide.

Epigenetic mechanisms such as DNA methylation interfere with gene transcription (“gene silencing”) or enable gene transcription (“gene turned on”) (Mill, 2011; Szyf & Bick, 2013). Moreover, epigenetic processes are responsive to changes in the environment (Meaney, 2010). Furthermore, these processes may be reversible, depending on the gene and location of methylation sites. Environmental influences are modulated by sensitive periods in development, when neurobiological circuitry is particularly responsive to experience and plasticity is most accessible (Cicchetti, 2015).

Although they are often long lasting, some epigenetic modifications may be transmitted across generations (Gapp, von Ziegler, Tweedie-Cullen & Mansuy, 2014; Mill, 2011; Roth, 2013; Szyf & Bick, 2013). Research findings document the utility of peripheral DNA methylation measures (Bick, 2012; Cicchetti et al., 2016a; Szyf & Bick, 2013). Methylation is the best investigated and most stable form of epigenetic modification involved in “gene silencing.” Consequently, DNA methylation is usually associated with decreased gene DNA expression. DNA methylation predominantly takes place at discrete CpG sites in the genome regions where cytosine nucleotides occur next to guanine nucleotides (Cecil, Walton, & Viding, 2015).

The glucocorticoid receptor (GR) gene, also known as NR3C1, is the receptor that binds with cortisol and other glucocorticoids. Early adversity and methylation of NR3C1 has been the focus of much epigenetic research (for examples, see reviews by Daskalaski & Yehuda, 2014; Palma-Gudiel, Cordova-Palomera, & Leza, 2015; Turecki & Meaney, 2016; and Tyrka

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et al., 2016). Negative early-life environments have been found to be associated with hypermethylation of NR3C1 exon 1_F promoter in 70% of animal studies and 89% of human early life adversity studies (Turecki & Meaney, 2016). In addition, all of the human parental stress investigations that examined NR3C1 at exon 1_F were found to be characterized by hypermethylation (Turecki & Meaney, 2016).

Epigenetic investigations have demonstrated that stress exposure in childhood is linked with methylation of NR3C1 in adults and children. For example, Tyrka et al., (2015) have found hypermethylation of NR3C1 at the exon 1_F promoter in association with early child maltreatment in pre-school-age children from low socioeconomic backgrounds. Likewise, in a study of 11–14 year old children, Romens, McDonald, Svaren, & Pollak (2015) also found that children who experienced physical maltreatment had greater methylation within exon 1_F in the NR3C1 promoter region of the gene in comparison to nonmaltreated children.

Moreover, Parade et al., (2016) found that pre-school-aged children who had been maltreated within the past six months exhibited methylation of NR3C1 at exon 1_D and 1_F that was positively correlated with internalizing, but not with externalizing behavior problems. The findings of Parade and colleagues (2016) provide corroborative evidence that early adversity is associated with methylation of NR3C1, an important regulator of the HPA axis. Perroud et al., (2011) also discovered increased methylation of NR3C1 in adults with a history of child maltreatment. Interestingly, child sexual abuse, the number of maltreatment subtypes, and the severity of abuse and neglect all were associated with NR3C1 hypermethylation (Perroud et al., 2011).

Studies investigating the outcomes of NR3C1 methylation have focused on psychopathology and behavioral problems. Although most studies suggest a link between increased methylation and internalizing symptomatology (see Dadds et al., 2015; Dammann et al., 2011; Parade et al., 2016; van der Knapp, et al., 2015; Wang et al., 2017; Yehuda et al., 2014), Heinrich et al., (2015) found that NR3C1 hypomethylation was associated with externalizing problems. Specifically, Heinrich et al., (2015) found that the group of young adults with a lifetime diagnosis of an externalizing disorder exhibited significantly lower NR3C1 methylation levels than the depressive disorder group and the healthy controls. Heinrich et al. (2015) interpreted the lower methylation levels in NR3C1 as a possible mechanism through which the differential development of externalizing disorders, as opposed to depressive disorders, may take place.

The studies reported in the literature suggest that methylation of NR3C1 may be a mechanism underlying the development of psychopathology among adults and children who experience early adversity (Tyrka et al., 2016). In the present epigenetic investigation, we examined the effect of various dimensions of child maltreatment (including the developmental timing of maltreatment, the number of maltreatment subtypes, and the chronicity of maltreatment) on methylation of the glucocorticoid receptor (GR) gene, NR3C1. In addition, we investigated the association between NR3C1 methylation and child outcomes. Our hypotheses are delineated below.

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NR3C1 Hypotheses

1. Maltreated children will evidence significant hypermethylation of exon 1_F of the NR3C1 gene compared to nonmaltreated children.

2. The developmental timing of children's maltreatment experience will influence NR3C1 methylation such that those children with early onset maltreatment will be significantly hypermethylated compared to their late onset maltreated and nonmaltreated peers.

3. The more maltreatment subtypes a child has experienced, and the more chronic the maltreatment experience, the greater the hypermethylation of NR3C1.

4. Hypermethylation of the NR3C1 gene will be associated with increased risk for a number of negative psychological outcomes and will mediate the effect of child maltreatment on these outcomes.

Method

Participants

Participants included 534 children who attended a research summer camp program for school-aged low-income maltreated (n =285) and nonmaltreated children (n =249). Children were on average 9.41 years old (SD = 0.88) and approximately half were female (n=259, 48.5%). The sample was racially and ethnically diverse (61.2% Black, 9.9% White, 20.6% Latino, and 8.2% biracial or other race). Informed consent was obtained from parents of maltreated and nonmaltreated children for their child's participation in the summer camp program and for examination of any Department of Human Services (DHS) records pertaining to the family.

Children in the maltreated group were recruited through a DHS liaison who examined Child Protective Services reports to identify children who had been maltreated and/or were part of a family with a history of maltreatment. Children living in foster care often experience early and extreme maltreatment. They were not recruited for the current investigation to reduce heterogeneity among the maltreated sample. The DHS liaison contacted eligible families and explained the study. Parents who were interested in having their child participate provided signed permission for their contact information to be shared with project staff. These families were representative of those receiving services through DHS. Comprehensive reviews of all DHS records for each family were conducted. Maltreatment information was coded by trained research staff and a clinical psychologist, using the Barnett, Manly, and Cicchetti (1993) nosological system for classifying child maltreatment. Coding is based on all available information and does not rely on DHS determinations.

Because maltreating families primarily have low socioeconomic status (National Incidence Study – NIS-4; Sedlak et al., 2010), nonmaltreating families were recruited from those receiving Temporary Assistance to Needy Families (TANF) in order to ensure socioeconomic comparability between maltreated and nonmaltreated families. A DHS liaison contacted eligible nonmaltreating families and described the project. Parents who were interested in participating signed a release allowing their contact information to be

given to project staff for recruitment. The families were recruited as nonmaltreated families after comprehensive DHS record searches confirmed the absence of any documented child maltreatment. Families who received preventative DHS services due to concerns over risk for maltreatment were not included within the nonmaltreated comparison group. In order to further verify a lack of DHS involvement, trained research assistants interviewed the mothers of children recruited for the nonmaltreatment group using the *Maternal Child Maltreatment Interview* (Cicchetti, Toth & Manly, 2003) and reviewed records in the year following camp participation to assure that all information had been assessed.

Maltreated and nonmaltreated children were compared on a number of demographic characteristics (see Table 1). Groups did not differ in terms of maternal marital status ($\chi^2(1, N=531)=1.21$, p=n.s.), maternal age ($t(530)=-1.28$, p=n.s.), child age ($t(533)=-1.215$, p=n.s.), and family history of receiving public assistance ($\chi^2(1, N=530)=.86$, p=n.s.). Nonmaltreated children were more likely to be African-American ($\chi^2(1, N=534)=8.86$, $p<.01$) and female ($\chi^2(1, N=534)=6.74$, $p<.05$).

Procedures

Day Camp Procedures—Maltreated and nonmaltreated children were randomly assigned to groups of ten same-sex and same-age peers. Within these groups five children were maltreated and five were nonmaltreated. Each group was led by three trained camp counselors who were unaware of child maltreatment status and study hypotheses. Children participated in recreational activities throughout the week. After child assent was obtained, children participated in research assessments conducted by trained research assistants. The intensive staff to child ratio allowed for counselors to closely interact with children. During the 35 hours of interaction throughout the camp week, counselors got to know children well. DNA samples via saliva also were obtained from children, as described below. All research assistants were unaware of child maltreatment status and study hypotheses. (For camp procedures see Cicchetti & Manly, 1990).

Measures

Maltreatment classification system *(MCS;* Barnett, Manly, & Cicchetti, 1993; Cicchetti & Barnett, 1991)—The Maltreatment Classification System (MCS) is designed to assess individual children's maltreatment experiences. The MCS utilizes DHS records to make independent determinations of maltreatment. The MCS classifies the subtypes that each child experienced, frequency of occurrence, subtype severity, and developmental periods of occurrence in order to designate the recency, onset, and chronicity of maltreatment. Subtypes of maltreatment include neglect, emotional maltreatment, physical abuse, and sexual abuse. *Neglect* refers to failure to provide for the child's basic physical needs for adequate food, clothing, shelter, and medical treatment. Neglect also includes lack of supervision, moral-legal neglect, and educational neglect. *Emotional maltreatment* involves extreme thwarting of children's basic emotional needs for psychological safety and security. Examples include belittling and ridiculing the child, extreme negativity and hostility, child abandonment, suicidal or homicidal threats, and extreme negativity and hostility. *Physical abuse* involves nonaccidental physical injury to the child such as bruises, welts, burns, chocking, and broken bones. *Sexual abuse* involves attempted or actual sexual

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contact between the child and caregiver for purposes of the caregiver's sexual satisfaction or financial benefit. Examples of sexual abuse range from exposure to pornography or adult sexual activity to sexual touching and fondling to forced intercourse with the child.

The MCS has demonstrated reliability and validity in classifying maltreatment in a number of studies (Bolger, Patterson, & Kupersmidt, 1998; Dubowitz, Pitts, Lintrownik, Cox, Runyan, et al., 2005, English, Upadhyaya, Litrownik, Marshall, Runyan, et al., 2005, Manly, 2005; Smith & Thornberry, 1995). DHS records were coded using the MCS by trained research staff and a clinical psychologist. All coders achieved adequate reliability before coding records used for the study. Kappas for the presence of each of the maltreatment subtypes ranged from .90 to 1.00; intraclass correlations for severity ratings of individual subtypes of maltreatment ranged from .83 to 1.0.

Regarding maltreatment subtype, 75.4% of the maltreated children experienced neglect, 62.5% experienced emotional maltreatment, 28.4% physical abuse, and 8.8% experienced sexual abuse. Consistent with other samples of maltreatment, the majority of children in this study experienced more than one subtype of maltreatment. Specifically, 58.9% of maltreated children had experienced two or more subtypes of maltreatment (M=.93 SD = 1.02). Developmental timing of maltreatment was determined by an investigation of discrete developmental periods, including infancy, toddlerhood, preschool, early school age, and later school age. This information was used to calculate the number of developmental periods in which each child experienced maltreatment. Nonmaltreated children were coded 0 in this chronicity variable. Among maltreated children, 57.2% experienced maltreatment during 1 developmental period (M=1.57 SD = .75). Developmental timing information was also used to determine the age of onset of maltreatment. The following groups were created: 0=nonmaltreated, 1=early onset maltreatment (maltreatment originating in infancy or toddlerhood), 2=later onset maltreatment (maltreatment originating in the preschool years or later). In the current investigation, 42.8% of the maltreated children had an age of onset in infancy or toddlerhood.

DNA methylation—Salivary DNA samples were collected from participants using Oragene DNA collection tubes (DNA Genotek®). DNA was later isolated from 450 μl of Oragene-DNA/saliva solution using the PrepIT-L2P protocol. The diluted DNA samples were submitted to the BioMedical Genomics Center (BMGC) at the University of Minnesota for quality analysis and testing of whole genome methylation analysis using the HumanMethylation450 BeadChip (Illumina). The samples were assayed for quality by determining the concentration, using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Item #P7589) and Real Time PCR (TaqMan) quantification of human DNA concentration. All samples passed BMGC quality control standards and a normalized 0.5 μg human DNA for each participant was utilized in the subsequent methylation analyses.

Each 0.5 μg DNA sample was subjected to bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research, D5003) which converts unmethylated Cytosine bases to Uracils. This method utilizes the methyl group attached to a Cytosine as a protecting group to deamination and subsequent conversion to a Uracil. After bisulfite conversion, the total amount of DNA was increased by methylation specific amplification (MSA) using a whole

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genome amplification process which copies the converted Uracils to Thymine bases. The DNA was then enzymatically fragmented in an end-point fragmentation process.

Microarray processing and analysis of the Illumina Infinium HumanMethylation 450K BeadChip was also done by the University of Minnesota's BMGC. This covers over 485,000 individual sites with single nucleotide resolution of CpG sites both inside and outside CpG islands. The 450K BeadChip offers comprehensive genome-wide coverage including 99% of RefSeq genes with high quality by using more than 600 negative controls. Bisulfite converted samples were then hybridized to these BeadChips followed by washing and staining per protocols prescribed by Illumina. The microarray bead chips were then imaged using a HiScan SQ system.

The fluorescence data were subsequently analyzed using the Methylation Module v1.9.0 of the GenomeStudio software package v2011.1 (Illlumia). All data were background corrected and negative control normalized producing average beta values. This average beta value represents the relative quantity of methylation at an individual site ranging from 0 to 1 (unmethylated to completely methylated). Tests that produced different results from technical replicates, originating from same source individual and collection type, of study participant samples were identified as poor and removed from subsequent analyses. This was accomplished by using differential methylation analysis of replicate sample average beta. Criteria for exclusion of CpG loci based on lack of precision within technical replicates was identified by selecting sites with |DiffScore| > 13, which is equivalent to a $p<0.01$. Tests corresponding to these suspect loci (N=5,244), those tests with p-values of greater than 0.01 (N=1,603), and SNP tests (N=65) were excluded (N=6,638, 1.4 %). Beta values were analyzed using principle component analysis (PCA) in Partek Genomics Suite, Partek Inc. software. Review of the data distribution identified 2 samples as outliers which were subsequently removed from further analyses.

DNA collection, extraction, and genotyping—Saliva was collected using the Oragene-DNA collection kit from DNA genotek inc. then DNA isolated using the manufactures protocol for 0.5 ml of Oragene-saliva material. The DNA was diluted to a working concentration and genotyping was completed in Cicchetti's University of Minnesota molecular biology lab for the polymorphism in the NR3C1 gene commonly known as BClI (NT_029289.11:g.3942244T>C) using previously reported primer and probe sequences (Wust et al., 2004). Individual allele determinations were made using TaqMan Genotyping Master Mix (Applied Biosystems, Catalog 4371357) with amplification on an GeneAmp 9700 (Applied Biosystems) and analyzing the endpoint fluorescence using a Tecan M200 and data analyzed with JMP 8.0 (SAS, Inc.). Human DNA from cell lines was purchased from Coriell Cell Repositories for all representative genotypes in duplicate and genotypes confirmed by sequencing using DTCS on an ABI 3130xl. These and no template controls were run alongside study samples representing 9% of the total data output. Any samples that were not able to be genotyped to a 95% or greater confidence were repeated under the same conditions. The call rate for the GR SNP was 99.8%. GR SNP distribution did not deviate from Hardy-Weinberg equilibrium (χ^2 (1) = .04, p=n.s.). The frequency distribution of the GR SNP was as follows: CC=56.6% CG=37.1%; GG=6.4%. Genotypes CG and GG were combined in these analyses because of the low frequency of GG.

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Maltreated and nonmaltreated children did not differ in GR SNP distribution (χ^2 (1) = .05, p=n.s.). Also, there was no difference in GR SNP distribution between African-American children and non-African-American children (χ^2 (1) = 1.18, p=n.s.).

Ego undercontrol—Children's ego undercontrol was measured using the California Child Q-Set (CCQ; Block & Block, 1980) which consists of 100 items about children's personality, social, and cognitive functioning. At the end of each week, following intensive observations and interactions with the child, two camp counselors independently completed the CCQ. Items were sorted into nine categories ranging from most to least descriptive of a particular child, according to a forced-choice method. Interrater agreement based on average intraclass correlations ranged from .80 to .87.

To generate ratings of ego control, the Q-Set descriptions were correlated with the criterion sorts for prototypical children demonstrating ideal levels of ego control. Each child's correlation with the criterion was calculated, and those correlations were averaged to yield an ego control score for each child. The resulting scores represented how similar or different the individual child was to the prototypical ego controlled child. High scores indicate high ego undercontrol and low scores indicate high ego overcontrol.

Emotional lability-negativity—The Emotion Regulation Checklist (ERC; Shields & Cicchetti, 1997, 1998) is a 24 item measure that may be completed by adults familiar with a child, including camp counselors, teachers and parents. The ERC includes both positively and negatively weighted items regarding emotionality and emotion regulation. The ERC yields two subscales: emotion regulation and emotional lability/negativity. Emotional lability/negativity was used in the current study. This subscale is composed of items related to mood swings, angry reactivity, emotional intensity, and dysregulated positive emotions. Two camp counselors completed the ERC after a week of intensive interactions and observations with the child. Inter-rater reliability for the emotional lability/negativity scale was .80.

Externalizing and internalizing behavior—Children's internalizing and externalizing symptoms were assessed at the end of the week through completion of the Teacher Report Form (TRF; Achenbach, 1991). The TRF is an extensively used and well-validated measure of a wide range of child symptomatology. On the TRF, camp counselors rated the frequency of occurrence of a list of problem behaviors that form the broadband externalizing factor (e.g., aggressive behaviors and delinquent behaviors) and the broadband internalizing factor (e.g., withdrawal, somatic complaints, anxiety-depression). Children were each rated by two camp counselors, and the scores for internalizing symptoms were averaged across raters, as were the scores for externalizing symptoms. Interrater reliabilities based on average interclass correlations among pairs of raters ranged from .70 to .88 for internalizing symptoms and .83 to .91 for externalizing symptoms.

Depressive symptoms—Children self-reported their depressive symptoms in the past two weeks using the Children's Depression Inventory (CDI; Kovacs, 1982, 1992), which is widely used for this purpose among school-aged children. Validity of the measure has been

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well established (Kovacs, 1982, 1992) and internal consistency for the total score has ranged from 0.71 to 0.89.

Anxiety symptoms—Child anxiety symptoms were assessed using the Revised Child Manifest Anxiety Scale (RCMAS; Reynolds & Richmond, 1985). The RCMAS is a 37-item self-report measure used to assess anxiety in children and adolescents aged 6 to 19. Response options are "yes" or "no" and items are summed for a Total Anxiety score. Reliability and validity of the scale have been demonstrated (Reynolds & Richmond, 1985).

Data Analytic Plan—Prior to conducting analyses, beta values for the 3 CpG sites in the NR3C1 exon 1_F (cg04111177, cg15910486, cg18068240) were transformed using the M-value method. M-values have been shown to be more statistically valid for differential analyses of methylation levels compared to beta values (Du, Zhang, Huang, Jafari, Kibbe, Hou, & Lin, 2010). The first set of analysis of variances (ANOVAs) examined the effect of maltreatment on NR3C1 methylation for each individual CpG site and also the mean NR3C1 methylation score and included GR genotype variation as well as the interaction of methylation and genotype. To conduct a comprehensive investigation of the effect of dimensions of child maltreatment on NR3C1 methylation at this region, we tested the effect of maltreatment status (0=nonmaltreated, 1=maltreated, as described above), the number of maltreatment subtypes experienced, the number of developmental periods in which maltreatment occurred, and the age of onset of maltreatment.

Preliminary analyses indicated that child age was not significantly correlated with mean NR3C1 methylation or individual CpG sites. Moreover, t-tests indicated the lack of significant differences between boys and girls on methylation (mean score and individual CpG sites), and lack of significant differences between African-American and non-African-American children on methylation (mean score and individual CpG sites). Therefore, for analyses examining associations between maltreatment parameters and methylation, these variables were not included as covariates. GR genotype variation was included as a covariate in all analyses.

To examine associations between NR3C1 methylation and child outcomes, a series of partial correlations were tested. Preliminary analyses indicated that older children evidenced less ego undercontrol (r=−.10, p=.02), less emotional lability/negativity (r=−.15, p=.001), less depressive symptoms (r=−.17, p<.001) and less overall internalizing symptoms (r=−.10, p=.03). Additionally, boys were viewed as having higher ego undercontrol (t(527)=−2.02, p=.04) and higher emotional lability/negativity (t(527)=−5.09, p<.001) compared to girls. Boys self-reported higher depressive symptoms (t(514)=−2.51, p=.01) and anxiety symptoms (t(516)=−3.03, p=.003) compared to girls. Preliminary analyses also indicated that African-American children evidenced significantly higher externalizing behaviors (t(528)=−2.38, p=.02) compared to non-African-American children. There were no other racial differences found on child outcomes. Therefore, child age, sex, and race were included in analyses with outcome variables when appropriate. GR genotype was included in all analyses.

A path analysis was estimated in Mplus Version 7.11 (Muthen & Muthen, 1998–2013) to examine whether NR3C1 exon 1_F methylation represents a mechanism by which

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maltreatment experiences affect the following outcomes: children's overall externalizing symptoms (counselor-report), ego undercontrol (counselor-report), emotional lability-negativity (counselor-report), depressive symptoms (child self-report), anxiety symptoms (child self-report), and overall internalizing behavior symptoms (counselor-report). We used full information maximum likelihood (FIML) estimation to handle missing data. FIML handles missing data under the assumption that the data were missing at random (MAR). Methylation values are often not normally distributed. To address the non-normality, the maximum likelihood (MLR) estimator in Mplus was employed. Model fit was evaluated with the maximum likelihood chi-square statistic, comparative fit index (CFI), root mean square error of approximation (RMSEA), and the standardized root mean squared residual (SRMR). CFI values greater than 0.95, RMSEA values less than 0.06, SRMR values less than .08, and a nonsignificant chi-square statistic were used as indicators of good model fit (Hu & Bentler, 1999; Yu & Muthen, 2002). RMediation was used to test the indirect effect of child maltreatment on the outcomes via NR3C1 methylation (Tofighi & MacKinnon, 2011). 95% asymmetric confidence limits that do not include the value zero indicate significant mediation.

Results

Maltreatment and NR3C1 Exon 1_F Methylation

Maltreatment Status—A series of analyses of variance (ANOVAs) were conducted to examine the effect of maltreatment status on each of the three NR3C1 CpG sites, as well as the mean score. GR genotype variation and the interaction of maltreatment status and GR genotype were also included in the model. See Table 2 for results. Results indicated that for CpG sites cg15910486 ($F(1)=4.53$, $p=.034$) and cg18068240 ($F(1)=6.16$, $p=.013$), as well as mean NR3C1 exon 1_F methylation ($F(1)=5.58$, $p=.019$), maltreated children evidenced hypermethylation compared to nonmaltreated children. No differences were found between maltreated and nonmaltreated children on methylation at individual CpG site cg04111177 ($F(1)=.62$, p=n.s.).

No methylation differences between genotype groups were found for CpG sites cg15910486 ($F(1)=.28$, p=n.s.), cg18068240 ($F(1)=.03$, p=n.s.), or the mean NR3C1 exon 1_F methylation ($F(1)=.58$, p=n.s.). For CpG site cg04111177, results indicated that individuals with CC genotype evidenced significant hypomethylation ($M=-4.10$, $SE=.01$) compared to individuals with CG/GG genotype ($M=-4.05$, $SE=.02$; $F(1)=5.27$, $p=.03$). None of the maltreatment by genotype interactions were significant in predicting methylation for the 3 individual CpG sites or the mean score.

Developmental Timing of Maltreatment—Next, we tested the effect of the developmental timing of maltreatment on NR3C1 methylation. The following 3 groups were included: 0=nonmaltreated, 1=early onset maltreatment (i.e. infancy or toddlerhood onset), 2=later onset maltreatment (i.e. preschool years or beyond). GR genotype was included in all models, as was the interaction of developmental timing and GR genotype. Results of an ANOVA (Table 2) indicated a significant difference between developmental timing groups on mean NR3C1 methylation ($F(2)=5.63$, $p=.004$). Bonferroni comparisons indicated that

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early onset maltreated children (*M*=−4.50, *SE*=.03) evidenced significant NR3C1 mean hypermethylation compared to nonmaltreated children (*M*=−4.63, *SE*=.02; p=.003). Nonmaltreated children and late onset maltreated children did not differ in NR3C1 greater mean methylation (*p*=n.s.), nor did early onset and late onset maltreated children differ (*p*=n.s.). The main effect of genotype and the interaction of genotype and maltreatment timing were nonsignificant.

These analyses were repeated for the 3 individual CpG sites. For site cg15910486, results indicated a significant difference between the 3 developmental timing groups on methylation at this site ($F(2)=3.96$, $p=.02$). Bonferroni comparisons indicated that early onset maltreated children (*M*=−3.30, *SE*=.03) did not differ from late onset maltreated children (*M*=−3.22, *SE*=.03; *p*=n.s.). Moreover, early onset maltreated children did not differ significantly from nonmaltreated children (*M*=−3.31, *SE*=.02; *p*=n.s.). Late onset maltreated children evidenced NR3C1 greater mean hypermethylation compared to nonmaltreated children ($p=.015$). The main effect of genotype and the interaction of genotype and maltreatment timing were nonsignificant.

For CpG site cg04111177, a trend-level difference was observed between groups ($F(2)=2.71$, $p=.068$). None of the Bonferroni comparisons reached statistical significance. Results indicated a significant difference between genotype groups on methylation at this site ($F(1)=7.27$, $p=.01$). Individuals with CC genotype evidenced significant hypomethylation (*M*=−4.10, *SE*=.01) compared to individuals with CG/GG genotype (*M*=−4.04, *SE*=.02). The interaction of maltreatment timing and genotype was nonsignificant.

Lastly, for CpG site cg18068240, results indicated a significant difference between groups ($F(2)=8.63$, $p<.001$). Bonferroni comparisons indicated that early onset maltreated children (*M*=−6.16, *SE*=.08) evidenced significant NR3C1 mean hypermethylation compared to nonmaltreated children (*M*=−6.56, *SE*=.06; p<.001). Moreover, early onset maltreated children also evidenced NR3C1 mean hypermethylation compared to later onset maltreated children (*M*=−6.51, *SE*=.07; $p=.004$). Nonmaltreated children and late onset maltreated children did not differ in NR3C1 mean methylation (*p*=n.s.). The main effect of genotype and the interaction of genotype and maltreatment timing were nonsignificant.

Number of Maltreatment Subtypes—Partial correlations were examined to determine the association between the number of maltreatment subtypes and NR3C1 methylation, controlling for GR genotype variation (Table 3). Results indicated that more maltreatment subtypes were related to mean NR3C1 hypermethylation ($r=.10$, $p=.03$). Higher levels of maltreatment subtypes were also related to NR3C1 hypermethylation at the following individual sites: CpG cg15910486 ($r=.10$, $p=.02$) and CpG cg18068240 ($r=.09$, $p=.04$). The number of maltreatment subtypes experienced was unrelated to methylation at CpG site cg04111177 ($r=-.06$, *p*=n.s.).

Maltreatment Chronicity—We next investigated the relation between the number of developmental periods in which maltreatment was experienced (chronicity) and NR3C1 methylation, again controlling for GR genotype variation (Table 3). Results indicated greater chronicity was associated with mean NR3C1 hypermethylation ($r=.11$, $p=.01$) and

hypermethylation at individual site CpG cg18068240 (r=.13, p=.004). Chronicity was unrelated to NR3C1 methylation at CpG sites cg15910486 (r=.03, p=n.s.) and cg04111177 (r=−.04, p=n.s.).

NR3C1 Exon 1_F Methylation and Child Outcomes

Partial correlations, controlling for child age, sex, race (African-American versus other), and GR genotype, were examined to determine the association between mean NR3C1 methylation and child outcomes (Table 4). Results indicated that NR3C1 mean hypermethylation was related to higher levels of ego undercontrol (r=.10, p=.04), higher levels of emotional lability-negativity (r=.10, p=.02), and greater externalizing behavior symptoms (r=.09, p=.04). NR3C1 hypermethylation was also associated with higher levels of child-reported depressive symptoms (r=.10, p=.03). NR3C1 methylation was not associated with child-reported anxiety symptoms (r=.06, p=n.s.) and overall counselor-reported internalizing behavior symptoms (r=−.01, p=n.s.).

Partial correlations also were tested, controlling for child age, sex, race, and GR genotype, to determine associations between individual NR3C1 CpG sites and child outcomes. An examination of individual CpG sites within the 1_F exon region indicated that hypermethylation at CpG site cg18068240 was associated with higher ego undercontrol (r=.11, p=.02), higher emotional lability-negativity (r=.12, p=.01), and higher externalizing symptoms (r=.11, p=.02). Hypermethylation at cg18068240 was also related to more depressive symptoms at a trend-level (r=.08. p=.078). Methylation levels at CpG sites cg04111177 and cg15910486 were not associated with the outcomes.

NR3C1 Exon 1_F as a Mediator

To examine the role of NR3C1 exon 1_F methylation in the relation between child maltreatment and child outcomes, path analysis was conducted. Number of maltreatment subtypes, age, sex, race, and GR genotype were modeled as exogenous variables. Mean NR3C1 methylation was modeled as a mediator and the following variables were included as endogenous variables: ego undercontrol, emotional lability-negativity, overall externalizing symptoms, depressive symptoms, anxiety symptoms, and overall internalizing symptoms. In a preliminary model, paths from child age, sex, and race to the mediator and all outcomes were specified. Those that were not statistically significant were trimmed from the final model. Paths from GR genotype to the mediator (NR3C1 methylation) and outcomes were estimated.

The final model fit the data well ($\chi^2(12)$=18.79, p=.09, CFI=.99, RMSEA=.03, SRMR=.03). Results indicated that more maltreatment subtypes were predictive of higher levels of ego undercontrol (b=.09, SE=.05, p=.04), higher levels of emotional lability-negativity (b=.19, SE=.04, p<.001), and higher levels of overall externalizing behavior symptoms (b=.19, SE=.05, p<.001). Greater number of maltreatment subtypes were also predictive of more depressive symptoms (b=.13, SE=.04, p=.002), and higher levels of overall internalizing behavior symptoms (b=.10, SE=.04, p=.03). More maltreatment subtypes was predictive of greater anxiety symptoms at a trend-level (b=.08, SE=.04, p=.05).

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Older children reported fewer depressive symptoms (b=−.14, SE=.03, p<.001) and were rated by counselors as having fewer internalizing behavior problems (b=−.14, SE=.04, p<.001) and less emotional negativity-lability (b=−.08, SE=.02, p<.001). Boys had higher levels of ego undercontrol (b=.07, SE=.02, p=.002), emotional negativity-lability (b=.23, SE=.02, p<.001), more depressive symptoms (b=.10, SE=.04, p=.02) and more anxiety symptoms (b=.13, SE=.04, p=.004). GR genotype variation did not significantly uniquely predict NR3C1 methylation or any of the outcomes.

Consistent with the above analyses, children who experienced more maltreatment subtypes also evidenced mean NR3C1 hypermethlyation at exon 1_F (b=.10, SE=.04, p=.02), over and above the effect of GR genotype. NR3C1 hypermethylation was associated with higher ego undercontrol (b=.09, SE=.04, p=.04), higher emotional lability-negativity (b=.09, SE=.04, p=.03), and higher overall externalizing symptoms (b=.09, SE=.04, p=.04). NR3C1 was not uniquely associated with depressive symptoms, anxiety symptoms, or overall internalizing symptoms.

To test whether NR3C1 represents a mechanism by which child maltreatment affects various child outcomes, RMediation was used (Tofighi & MacKinnon, 2011). 95% asymmetric confidence limits that do not include the value zero indicate significant mediation. Results did not support significant mediation of NR3C1 methylation in the relation between child maltreatment and ego undercontrol (UCL= .006; LCL= 0), emotional lability-negativity (UCL= .011; LCL= 0), overall externalizing symptoms (UCL=.204; LCL=−.002), depressive symptoms (UCL=.125; LCL= −.01), anxiety symptoms (UCL= .239; LCL=−.027), or overall internalizing symptoms (UCL= .065; LCL=−.076).

Discussion

Results indicated significant hypermethylation of the NR3C1 exon 1_F mean among the maltreated children compared to the nonmaltreated children. These findings are congruent with the conclusions drawn in a number of prior reviews of animal and human studies that demonstrate a link between early adversity and hypermethylation of NR3C1 (Daskalaski & Yehuda, 2014; Palma-Gudiel et al., 2015; Turecki & Meanery, 2016; Tyrka et al., 2016). We advance the literature by demonstrating the importance of investigating maltreatment parameters (developmental timing of maltreatment, the number of maltreatment subtypes, and the chronicity of maltreatment) in relation to NR3C1 methylation. To our knowledge, the only study conducted to date that has examined various dimensions of maltreatment was the study by Perroud et al., (2011) which employed a sample of adults with borderline personality disorder. These investigators found that hypermethylation was associated with a greater number of maltreatment subtypes and greater severity of childhood abuse and neglect. The present investigation expands upon this work by employing a large representative sample of children with maltreatment experiences, documented prospectively from the coding of Department of Human Services record data and not retrospectively through adult-self-report on the Child Trauma Questionnaire (CTQ) (Bernstein & Fink, 1998) with a matched comparison group of children, and by controlling for genotype in all analyses.

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Consistent with a growing literature on the negative consequences of child maltreatment experienced during the early years of life (Cicchetti, Handley, & Rogosch, 2015; Cicchetti, Rogosch, Gunner, & Toth, 2010; Curtis & Cicchetti, 2013; Dunn, McLaughlin, Slopen, Rosand, & Smoller, 2013; Kaplow & Widom, 2007; Manly, Kim, Rogosch, & Cicchetti, 2001), our results suggest that children who experience maltreatment during infancy and/or toddlerhood display significantly greater hypermethylation of the GR gene compared to nonmaltreated children. In addition, greater chronicity (the number of developmental periods in which maltreatment was experienced) was also related to higher methylation of NR3C1. Furthermore, the experience of more maltreatment subtypes also was related to higher hypermethylation. These findings are consistent with that of Parade and colleagues (2016) who discovered that higher adversity composite scores were linked with higher hypermethylation among maltreated preschool-age children.

With regards to associations between NR3C1 methylation and various childhood psychological outcomes, the results of the present investigation of maltreated children indicated that higher mean NR3C1 was related to the following negative outcomes: higher levels of ego undercontrol, higher levels of emotional lability-negativity, greater externalizing behavior symptoms, and higher depressive symptoms. The outcomes were based on adult counselor ratings of the children after 35 hours of observation in a camp setting and child self report (CDI). These results highlight the role of methylation of NR3C1 in the effects of child maltreatment on the development of emotion dysregulation and psychopathology. We did not find higher mean NR3C1 to be related to overall anxiety symptoms, or counselor observational ratings of overall internalizing symptoms.

Our results are partially consistent with the prior literature on NR3C1 and internalizing symptoms. Specifically, the investigation of Parade et al., (2016) found higher hypermethylation associated with higher internalizing scores among preschoolers, but not associated with higher externalizing scores. Developmental differences in samples may explain the disparate findings. Our sample had a mean age of 9.4 years, compared to the 4.2 year old mean of the Parade et al. (2016) sample.

Meaney and Szyf (2005) found that methylation leads to less GR transcription and functionality and increased risk for anxiety and depression. Likewise, in their study of 241 4–16 year old clinic-referred children, Dadds and colleagues (2015) also found some evidence for increased methylation associated with higher levels of externalizing symptoms; however, these results were only obtained in salivary DNA samples, but not in blood samples. van der Knapp et al. (2015) found NR3C1 methylation to be associated with risk for lifetime internalizing disorders. These results are consistent with animal models demonstrating that NR3C1 methylation is associated with anxiety-like behaviors (see Tyrka et al; 2016, for review).

The present investigation extends prior research by showing links with hypermethylation of NR3C1 and ego undercontrol and emotional lability/negativity, both of which are associated with underlying processes of psychopathology. We also extend the literature through demonstrating links with NR3C1 hypermethylation and externalizing symptoms. Contrary to our hypothesis, hypermethylation of NR3C1 did not mediate the effect of child maltreatment

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on these outcomes. Parade et al. (2016) demonstrated that NR3C1 methylation mediated the association between child maltreatment and childhood behavioral problems. It is important to note a number of methodological differences between the Parade et al. (2016) study and the present investigation. First, as described previously, the Parade et al. (2016) study employed a sample of preschool children and the current study used a sample of school-aged children with a mean age of 9. Also, Parade and colleagues (2016) examined NR3C1 methylation at exons 1_D and 1_F; the present study examined methylation of exon 1_F exclusively. Finally, the present study used both self-report and counselor-report measures of child internalizing and externalizing symptoms, as compared to the Parade et al. (2016) study which relied on a parent report measure.

Implications and Future Directions

Epigenetic mechanisms may serve as a target for intervention because of their reversibility. Prevention scientists could include DNA methylation and gene expression in the design of their interventions (e.g., at baseline, at end of intervention, at one-year follow-up) in order to evaluate the efficacy of the interventions on epigenetic mechanisms. These methylation assays may be conducted genome-wide or at the level of specific regions of candidate genes with known functional properties, such as NR3C1 (Szyf & Bick, 2013). DNA is either methylated or demethylated in response to environmental experiences. Reversibility of DNA methylation is essential for multi-level interventions whose goal is to reset epigenetic programming (Cicchetti, in press; Klengel, et al., 2013; Roberts, et al., 2015). The changes that define the outcomes/phenotype are not caused solely by inherited genetic polymorphisms, but by genotypic variation and epigenetic modifications (Cicchetti et al., 2016a, b; Mill, 2011, Szyf & Bick, 2013). Thus, it is conceivable that interventions may reverse DNA methylation and allay negative outcomes (Szyf & Bick, 2013; Toth, Gravener-Davis, Guild, & Cicchetti, 2013). With increasing advances in molecular biology, neurobiology, and a multiple-levels of analysis approach (Cicchetti & Gunnar, 2008), prevention science will be in a better position to develop a fuller understanding of the mechanisms underlying efficacious intervention.

Given the central role of NR3C1 in the stress response system, future research investigating the associations among NR3C1 methylation and cortisol regulation and immune system functioning among maltreated children will be critical. Moreover, we focused on child maltreatment given its prevalence and negative developmental consequences; however, future research examining other forms of childhood adversity and effects on NR3C1 methylation will be important. Finally, we examined links between NR3C1 methylation and child outcomes during the school-aged years. It will be informative to determine whether early maltreatment experiences are associated with NR3C1 methylation throughout adolescence and emerging adulthood.

In summary, the present study examined NR3C1 methylation among a sample of maltreated and nonmaltreated children and investigated links with a number of negative psychological outcomes. Findings show that children with early-onset maltreatment evidence significant NR3C1 hypermethylation compared to nonmaltreated children. More maltreatment subtypes experienced and more chronic maltreatment are both related to greater NR3C1

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hypermethylation. Importantly, hypermethylation of NR3C1 is linked with a number of negative child outcomes including greater emotional lability-negativity, higher levels of ego undercontrol, more externalizing behavior, and greater depressive symptoms. Together our results suggest that NR3C1 methylation is influenced by various child maltreatment experiences and that hypermethylation of NR3C1 may contribute to the development of psychopathology among children.

Acknowledgments

We are grateful for the research support provided by the Emerald Foundation, Inc., and the Jacobs Foundation to Dante Cicchetti, and the National Institute of Mental Health (R01-MH83979 to Dante Cicchetti).

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Table 1

Comparison of maltreated and nonmaltreated children on demographic characteristics

	Nonmaltreated (n=249)		Maltreated (n=285)	
	M (SD)	Percentage	*M (SD)*	Percentage
Child age (years)	9.37 (.85)		9.45 (.92)	
Child gender (% male) *		45.60		56.84
African-American **		68.00		55.39
Maternal age	33.64 (6.20)		34.35 (6.41)	
Receipt of public assistance		93.20		97.19
Single mother		64.37		60.00

* $p<.05$,

** $p<.01$

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Table 2

Maltreatment and NR3C1 methylation

	Mal n=285	Nonmal n=249	Test statistic	Nonmal n=244	EO mal n=121	LO mal n=160	Test statistic	Significant Bonferroni contrasts
NR3C1 mean	−4.55 (.02)	−4.63 (.02)	$F(1) = 5.58^{*}$	−4.63 (.02)	−4.50 (.03)	−4.60 (.03)	$F(2) = 5.63^{**}$	Nonmal<EO
cg04111177	−4.08 (.01)	−4.07 (.02)	$F(1) = .62$	−4.07 (.02)	−4.05 (.02)	−4.11 (.02)	$F(2) = 2.71^{\dagger}$	--
cg15910486	−3.25 (.02)	−3.32 (.02)	$F(1) = 4.53^{*}$	−3.31 (.02)	−3.30 (.03)	−3.22 (.03)	$F(2) = 3.96^{*}$	Nonmal<LO
cg18068240	−6.37 (.05)	−6.56 (.06)	$F(1) = 6.16^{*}$	−6.56 (.06)	−6.16 (.08)	−6.51 (.07)	$F(2) = 8.63^{***}$	Nonmal<EO, LO<EO

Notes: Mal=maltreated, Nonmal=nonmaltreated, EO mal= early onset maltreated, LO mal= late onset maltreated. Means are presented with standard errors in parentheses.

† p=.07,

* p<.05,

** p<.01,

*** p<.001

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Table 3

Partial correlations between maltreatment subtypes and chronicity and NR3C1 methylation, controlling for GR genotype variation

	NR3C1 mean	cg04111177	cg15910486	cg18068240
Maltreatment subtypes	.10 *	−.06	.10 *	.09 *
Maltreatment chronicity	.11 *	−.04	.03	.13 **

Notes:

* p<.05,

** p<.01

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Table 4

Partial correlations between NR3C1 methylation and outcomes

	EU	ELN	Ext	Dep	Anx	Int
NR3C1 mean	.10*	.10*	.09*	.10*	.06	.01
cg04111177	.05	.01	−.01	.07	.07	−.06
cg15910486	−.02	.01	.01	.05	.03	.02
cg18068240	.11*	.12*	.11*	.08†	.04	.01

Notes: Child age, sex, race, and GR genotype are controlled in correlations. EC=ego undercontrol, ELN=emotional lability-negativity, Ext=externalizing behavior, Dep=depressive symptoms, Anx=anxiety symptoms, Int=internalizing behavior.

† $p<.10$,

* $p<.05$.