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Regular Article

Maternal sensitivity and child internalizing and externalizing behavior: a mediating role for glucocorticoid receptor gene (*NR3C1*) methylation?

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Abstract

The early caregiving environment can have lasting effects on child mental health. Animal models suggest that glucocorticoid receptor gene (*NR3C1*) DNA methylation plays a mediating role in linking more responsive caregiving to improved behavioral outcomes by its impact on the stress regulatory system. In this longitudinal study, we examined whether children's *NR3C1* methylation levels mediate an effect of maternal sensitivity in infancy on levels of child internalizing and externalizing behavior in a community sample. Maternal sensitivity of 145 mothers was rated at infant age 5 weeks, 12 months, and 30 months by observing mother–infant interactions. Buccal DNA methylation was assessed in the same children at age 6 years and maternal-reported internalizing and externalizing behavior was assessed at age 6 and 10 years. Higher sensitivity at age 5 weeks significantly predicted lower DNA methylation levels at two *NR3C1* CpG loci, although methylation levels at these loci did not mediate an effect of maternal sensitivity on levels of child internalizing and externalizing behavior. Overall, the study provides evidence that maternal sensitivity in early infancy is associated with DNA methylation levels at loci involved in stress regulation, but the significance of this finding for child mental health remains unclear.

Keywords: externalizing; internalizing; maternal sensitivity; methylation; NR3C1

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Introduction

Mental health problems are one of the main causes of disability worldwide and typically emerge before adulthood (Global Burden of Disease Study 2013 Collaborators 2015; Solmi et al., 2021; Whiteford et al., 2013). As such, it is crucial that we improve our understanding of early life factors that modify children's risk of developing mental health problems. One such factor is maternal sensitivity – the extent to which a mother is able to appropriately identify and respond to her infant's physical and emotional needs and signals (Ainsworth et al., 1974). Less sensitive caregiving during infancy is associated with higher levels of childhood internalizing behavior (i.e., anxiety, depression, and withdrawal; Kok et al., 2013) and externalizing behavior (i.e., aggression, defiance, and impulsivity; Wang et al., 2013; van der Voort et al., 2014). Child internalizing and externalizing behaviors are, in turn, major predictors of later life psychopathology (Mathyssek et al., 2012; Reef et al., 2009). However, the pathways by which maternal sensitivity influences children's levels of internalizing and externalizing behavior are not yet fully understood and warrant further investigation in order to inform preventive interventions (Deans, 2020; Provenzi et al., 2019).

Maternal sensitivity may influence children's levels of internalizing and externalizing behavior through its effects on the developing stress regulatory system in early infancy. Specifically, sensitive caregiving provides infants with the external support they need to cope with emotional and physiological stress, and thus a model for infants to learn how to independently regulate stress (Bell & Ainsworth, 1972; Bowlby, 1980; Gianino & Tronick, 1988). On the other hand, insensitive caregiving – characterized by absent, noncontingent and/or intrusive responses to an infant's signals and behaviors – not only fails to provide infants with external regulation but may act as a source of stress in itself (Smeekens et al., 2007; Tronick, 1989). As such, infants that receive insensitive caregiving may be at risk for long-term stress dysregulation (Laurent et al., 2016), which could contribute to higher levels of internalizing behavior and externalizing

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behavior (Ruttle et al., 2011). In this way, insensitive caregiving may act as a risk factor for later life psychopathology, while sensitive caregiving may act as a protective factor by ensuring that children are able to appropriately respond to the stressors that they will inevitably face in their daily environment. A potential biological pathway for this association is through epigenetic processes such as DNA methylation, a relatively stable modification to nuclear DNA that occurs most commonly at cytosine-guanine dinucleotide (CpG) sites and can functionally regulate gene expression (Aristizabal et al., 2020). Epigenetic changes in response to sensitive caregiving may alter the function of the hypothalamic pituitary adrenal axis to enhance recovery from stress and reduce stress-related behaviors such as internalizing and externalizing behaviors (Berretta et al., 2021). The current study tested the feasibility of this pathway in children by examining whether differential DNA methylation levels at the glucocorticoid receptor (GR) gene (*NR3C1*), a key regulator of the HPA axis, mediate an association between maternal sensitivity in infancy and later levels of internalizing and externalizing behavior.

Research in rodents has implicated DNA methylation changes at *NR3C1* as a mediator of the effects of maternal responsivity on levels of stress-related behaviors, such as internalizing-like and externalizing-like behaviors. More sensitive caregiving behavior by rat dams (i.e., licking, grooming and arch-backed nursing) during the first week of life has been found to lead to long-term hypomethylation at exon 1_7 of the *NR3C1* promotor in the hippocampal tissues of their offspring (Weaver et al., 2004). In turn, *NR3C1* promotor hypomethylation is associated with increased hippocampal GR expression, and subsequently more modest HPA axis responses and less defensive, stress-related behaviors in adult rats (Weaver et al., 2004, 2005, 2006; van Hasselt et al., 2012). Given that higher maternal sensitivity has also been found to predict better HPA axis regulation in human infants (Albers et al., 2008; Blair et al., 2006), it is possible that a similar epigenetic pathway may link more sensitive caregiving with lower levels of child internalizing and externalizing behavior in humans.

To date, human studies have predominately focused on the effects of severely disrupted caregiving (i.e., child maltreatment) on DNA methylation and provide some preliminary evidence that *NR3C1* hypermethylation may link an adverse caregiving environment to later psychopathology (for a review see Wadji et al., 2021). However, relatively little attention has been given to typical variation in caregiving within community samples. To our knowledge, only two human studies have investigated the link between maternal sensitivity and *NR3C1* methylation, while no studies have considered the pathway from maternal sensitivity through *NR3C1* to subsequent stress-related behaviors. In one of the aforementioned studies, maternal insensitivity was associated cross-sectionally with higher methylation at the *NR3C1* 1f region in buccal-derived DNA of 5-month-old infants, although results differed based on infant sex and maternal depressive symptoms (Conradt et al., 2016, 2019). Notably, increased methylation at the 1f region – orthologous to the exon 1_7 in rodents – has been repeatedly linked to mental health problems in adult populations (for a review see Watkeys et al., 2018). In contrast, no association was found between maternal sensitivity at age 3–4 years and *NR3C1* methylation levels in whole blood DNA at age 6 in a prospective, longitudinal study (Dall'Aglio et al., 2020). The difference in findings between studies could be related to differences in methodology (e.g., rating scales for maternal sensitivity and methylation analysis method), but could also be related to a difference in the developmental timing of the measurement of maternal sensitivity (i.e., 5 months vs. 3–4 years). Both rodent and human studies support the likelihood that there is a sensitive period in early infancy (i.e., before three years of age) during which the caregiving environment may have a more significant impact on children's DNA methylation (Curley & Champagne, 2016; Dunn et al., 2019). However, the timing of exposure to sensitive versus insensitive caregiving in relation to *NR3C1* methylation has not been considered in prior human studies. Moreover, it remains unclear from existing studies whether associations between maternal sensitivity and *NR3C1* methylation in early life persist into middle childhood and whether they mediate an effect of maternal sensitivity on children's behaviors.

Prior research has established associations of *NR3C1* methylation with child internalizing and externalizing behavior. A number of cross-sectional studies have demonstrated that higher parent-reported internalizing behavior associates with higher methylation levels at the *NR3C1* 1f exon in children aged 3–16 years (Dadds et al., 2015; Gardini et al., 2022; Parade et al., 2016), and at the *NR3C1* 1d exon in children aged 3–5 years (Parade et al., 2016). Furthermore, in a longitudinal study, higher *NR3C1* promoter methylation in adolescents aged 15–16 years predicted higher internalizing symptoms three years later (van der Knaap et al., 2015). Evidence for associations of *NR3C1* methylation with externalizing behavior is less consistent. In a study of children aged 4–16 years, higher parent-reported externalizing behavior was cross-sectionally associated with higher salivary *NR3C1* methylation levels at the 1f exon (Dadds et al., 2015). Likewise, positive associations were found between methylation levels at the 1f exon and parent-reported oppositional defiant problems in 3-year-olds (Gardini et al., 2022). In contrast, a cross-sectional study of children aged 3–5 years found no significant associations of parent-reported externalizing behavior with *NR3C1* promoter methylation (Parade et al., 2016), whereas a longitudinal study reported that lifetime externalizing disorder – based on parent reports from ages 3 to 19 years – predicted lower *NR3C1* 1f exon methylation at age 19 years (Heinrich et al., 2015). Together these findings lend some preliminary support for a pathway from *NR3C1* methylation to child behavior. However, there is a paucity of longitudinal studies during childhood that test said pathway. Furthermore, most studies to date have been limited in scope to the 1f exon of the *NR3C1* promoter and have relied solely on parental reports of child internalizing and externalizing behavior.

To address the gaps in prior research, the current study investigated whether *NR3C1* methylation mediates an effect of early maternal sensitivity on levels of internalizing and externalizing behaviors in later childhood. First, we examined whether maternal sensitivity at three time points in early infancy (5 weeks, 12 months, and 30 months) predicted children's *NR3C1* methylation levels at age six. Second, we examined whether children's *NR3C1* methylation levels at age 6 mediated an association of maternal sensitivity with children's levels of internalizing and externalizing behavior at ages 6 and 10 years. Based on earlier rodent models, we hypothesized that 1) more sensitive caregiving would be associated with lower levels of *NR3C1* methylation at age 6, and 2) CpG loci with lower levels of *NR3C1* methylation at age 6 would partially mediate a negative association of sensitive caregiving with children's levels of internalizing and externalizing behavior at ages 6 and 10 years. For the sake of completeness, we also explored whether there were associations between *NR3C1* methylation levels at age 6 on all 25 assessed CpG loci and child internalizing and externalizing behaviors at age 6 and 10 years.

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Methods

Participant characteristics

Participants were mother–infant dyads recruited as part of an ongoing prospective study on the role of early environmental factors in infant and child development (BIBO project; Basal Influences on Child Development; see Beijers et al., 2010, 2011). Mothers were recruited during pregnancy in collaboration with midwife clinics located in or close to the cities of Nijmegen and Arnhem, The Netherlands. The project was approved by the Radboud University ethical committee of the social science faculty (ECG300107, SW2017-1303-49) and written informed consent was obtained from each participant on enrollment. The original maternal study sample reflected a healthy, nonclinical population of 193 mothers aged 21–42 years with the following inclusion criteria: a singleton uncomplicated pregnancy, no drug use, and no current physical or mental health problems. All infants had an uncomplicated birth and were born healthy with a 5-min APGAR score $\geq$7. From the original study sample, 148 children provided buccal epithelial cells at age six for genetic and DNA methylation analyses with their mother's informed consent. All parents in the current study sample described their child's ethnic background as Caucasian. Chi-square and Mann–Whitney U tests revealed no significant differences between dyads with and without child buccal samples in terms of child sex, child birthweight, maternal age at childbirth, maternal sensitivity, and child behavioral outcomes.

Design

Maternal caregiving was observed at three time points: infant age 5 weeks (at home), 12 months (in the laboratory), and 30 months (at home). Buccal cell samples were collected for (epi)genetic analysis from the same children using buccal swabs when they were 6 years old. Mothers reported on the internalizing and externalizing behavior of their child via questionnaire when the same children were 6 years old and 10 years old. Children also reported on their own internalizing and externalizing behavior via questionnaire at age 10 years.

Measures

Maternal sensitivity

When the infants were five weeks old, mother–infant dyads were visited at home and mothers were videotaped while bathing their infant as they would normally (as described by Jansen et al., 2010, and Beijers et al., 2020). Two trained independent coders rated videotapes of the whole bathing routine for *sensitivity* – that is, the extent to which the mother timeously and adequately responds to the infant's needs and signals – on a 9-point rating scale using the Ainsworth Maternal Sensitivity Scale (Ainsworth et al., 1978). Interrater reliability was excellent (Cohen's kappa = .90). Higher scores represent mothers who displayed more appropriate responses to their child's needs and signals, that is, higher maternal sensitivity. Of the mothers, 24.3% received a rating of three or lower, reflecting low to inadequate care (Helmerhorst et al., 2017).

When the infants were 12 and 30 months old, mother–infant interactions were videotaped during a joint semi-structured play session (as described by Beijers et al., 2020). Specifically, at infant age 12 months, mothers were asked to play with their infants using four toys (e.g., puzzle, books, and hand puppets) for 3 minutes each during a lab visit. At infant age 30 months, mothers were asked to play with their children using three toys (e.g., puzzle and blocks) for 4 minutes each during a home visit. The videotapes were rated by two independent observers using the Erickson scales (Erickson et al., 1985). For each mother–infant dyad a composite score for maternal sensitivity was computed by taking the mean of the seven-point subscales for *supportive presence* and *respect for autonomy* (Kok et al., 2013). *Supportive presence* refers to the extent a mother shows positive regard and emotional support to their infant, while *respect for autonomy* describes how far the mother refrains from interfering with the infant's desires, interests, or behavior during the task. The two measures were highly and positively correlated at age 12 months ($r = .62$, $p < .001$), and moderately and positively correlated at age 30 months ($r = .44$, $p < .001$). Interrater reliability was excellent for *supportive presence* at 12 months (intraclass coefficient [IC] = .95) and 30 months (IC = .91), and moderate for *respect for autonomy* at 12 months (IC = .70) and 30 months (IC = .70). Higher composite scores represent higher maternal sensitivity. Of the mothers, 22.9% received averaged ratings of three or lower when their infants were 12 months old, reflecting low to inadequate care, while all mothers scored above three when their infants were 30 months old, reflecting at least adequate care (Helmerhorst et al., 2017).

Internalizing and externalizing behavior

Child internalizing and externalizing behaviors were measured by maternal report at age six using the Dutch-version of the Child Behavior Checklist for ages 4–18 (CBCL/4-18; Achenbach, 2007). The CBCL/4-18 has good reliability in Dutch samples and is predictive of adult mental problems (Dekker et al., 2002; Roza et al., 2003). Items were rated from zero (not true as far as you know) to two (very true or often true), and included items such as, "Unhappy, sad, or depressed". Ratings were used to form two broad-band scales: *internalizing* (anxiety-depression, somatic complaints, and withdrawal subscales) and *externalizing* (delinquent and aggressive subscales). Continuous raw scores, ranging 0–66 for both internalizing and externalizing, were used for each broad-band scale, with higher scores reflecting a higher frequency of child internalizing or externalizing behaviors.

Child internalizing and externalizing behaviors were also measured at age 10 years by child self-report with the Strengths and Difficulties questionnaire (SDQ; Mieloo et al., 2014), to confirm if study outcomes were consistent across informants and symptom checklists. An externalizing score ranging 0–20 was formed from the sum of the conduct and hyperactivity subscales, while an internalizing score ranging 0–20 was formed from the sum of the emotional and peer problems subscale (Goodman & Goodman, 2009).

NR3C1 DNA methylation

Genomic DNA was extracted from buccal epithelial cells with the QIAamp DNA Mini Kit (Qiagen, Germany) and quantified using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific). The Zymo EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) was then used to bisulfite convert 750 ng of gDNA, before genome-wide DNA methylation was described using the Infinium EPIC array (850k array; Illumina, San Diego CA, USA) in accordance with manufacturer's guidelines. Preprocessing was performed with the *meffil* package in R (Min et al., 2018). Probes failed quality control if for more than 10% of the samples they had either: a detection p-value >.01, and/or a bead count less than three. Moreover, samples were removed during quality control if either: the reported sex did not match the methylation-predicted sex, more than 10% of the sample's probes had a detection p-value >.01, and/or more than 10% of sample's

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probes had a bead count less than three. Three samples failed quality control leaving a study sample of 145 children for the main analyses. Additionally, functional normalization (FN; Fortin et al., 2014) was used to reduce non-biological differences between probes. Methylation was estimated based on the ratio of methylation signal to overall signal at each CpG and expressed as β-values ranging from 0 to 1. For our analyses, we extracted values for 25 CpG loci located within the CpG island in the 5' untranslated region of the *NR3C1* gene (GRCh37/hg19 chr5:142,782,071 to chr5:142,785,071; Palma-Gudiel et al., 2015). Buccal epithelial cell content of the samples was estimated using the approach described by Smith and colleagues (Smith et al., 2015) and included as a covariate in the main analyses to adjust for cell heterogeneity (Ong et al., 2014).

Genetic covariates

The same buccal samples were genotyped using the Infinium Global Screening Array (Illumina, Inc.). During quality control, two participants were excluded due to missing single nucleotide polymorphism (SNP) rates higher than 5%. A principal component analysis-based approach was used to account for population stratification, which can confound the results of methylation analysis (Barfield et al., 2014). The first two genetic principal components (PC1 and PC2), which best described the population structure of the sample, were included as covariates in the main analyses.

Maternal mental health

Mothers' scores on the State-Trait Anxiety Inventory (STAI; Spielberger et al., 1983) and Edinburgh Postnatal Depression Scale (EPDS; Pop et al., 1992) at 37 weeks gestational age, and at infant ages 3 months, 12 months, and 30 months, were used to control for maternal anxiety and depression in post hoc sensitivity analyses given potential confounding effects of maternal mental health on associations of maternal sensitivity with *NR3C1* methylation and child behavior.

Data analysis

Analyses were performed in IBM SPSS version 24 and an alpha level of 0.05 was used to assess statistical significance unless otherwise specified. To handle outliers and overcome violation of normality, child internalizing and externalizing scores were log-transformed during data preparation; as CBCL scores range from zero and log0 is undefined, we added a value of one to all scores before the log transformation, that is, transformed score = log (original score + 1). The missing data, which are described in Table 1, were missing completely at random (Little's test: $\chi^2(214) = 219.93$, $p = .376$) and thus handled using listwise deletion. Buccal cell count, genetic PC1 and PC2, child sex, child birthweight and maternal age at child birth (as a proxy for socioeconomic status) were selected *a priori* as covariates based on previous research and included in all statistical models (Barfield et al., 2014; Liu et al., 2010; McDade et al., 2019; Mulligan et al., 2012; Ong et al., 2014; Yousefi et al., 2015). In the preliminary analysis, Pearson's and biserial-point correlations were used to test associations between study variables (i.e., maternal sensitivity at three time points, internalizing and externalizing behavior at two time points, methylation at 25 CpG loci, and the preselected covariates).

In the first step of the main analyses, for each of the 25 CpG loci, a multiple regression analysis was performed to test maternal sensitivity scores at 5 weeks, 12 months, and 30 months as predictors of methylation levels. Scores for maternal sensitivity at each time point were included as separate predictors within each model, thus allowing us to test the individual effect of maternal sensitivity at each time point on children's *NR3C1* methylation. Multiple regression is a suitable method for testing the association of repeatedly measured predictors on an outcome variable when the predictors are not highly correlated, that is, when statistical assumptions are not violated due to multicollinearity, and when data are missing completely at random (Ha et al., 2007; Lubin et al., 1997; Sánchez et al., 2011). In our data, maternal sensitivity scores were not highly correlated between time points at the whole sample level (see Table 1 for Pearson's correlation coefficients) nor were maternal sensitivity scores highly correlated within subjects over time (intraclass correlation = 0.07, based on an unconditional means model run in R version 4.0.3). After running the multiple regression analyses for each CpG loci, a false-discovery rate (FDR) correction ($q = 0.05$) was applied to control for multiple comparisons (Benjamini & Hochberg, 1995). CpG loci whose methylation levels were significantly predicted by maternal caregiving at any timepoint after correction were selected for the mediation analyses.

In the next step of the main analyses, methylation levels at CpG loci selected in the first step were tested as mediators of a possible relationship between maternal sensitivity and child internalizing and externalizing behavior. We performed a separate parallel mediation analysis for each outcome variable (i.e., internalizing behavior at age 6, internalizing behavior at age 10, externalizing behavior at age 6, and externalizing behavior at age 10) using multiple regression and bootstrapping procedures described by Hayes (2013). Each mediation analysis tested a total effect model of the relationship between maternal sensitivity scores and internalizing/externalizing behavior scores, and a direct effect model of the same relationship while additionally controlling for CpG loci methylation levels. Maternal sensitivity scores at all three time points and the preselected covariates were included as predictors in all models. Ninety-five percent bias-corrected confidence intervals and 1000 bootstrap resamples were then used to determine the significance of the indirect effect (i.e., the reduction in the effect of maternal sensitivity on internalizing/externalizing behavior when CpG loci methylation levels were included in the model). Confidence intervals that did not include zero were interpreted as statistically significant and indicative of partial mediation. As a sensitivity check, the mediation analyses were repeated with self-reported child internalizing and externalizing behavior scores at age 10 years as the dependent variable.

Additionally, Spearman's rank partial correlations were conducted to explore associations between methylation levels at individual CpG loci and child internalizing and externalizing behavior at both 6 and 10 years of age while controlling for the preselected covariates. Again, a false-discovery rate (FDR) correction ($q = 0.05$) was applied to control for multiple comparisons.

Finally, we conducted two post hoc analyses. First, in case of confounding between maternal mental health and maternal sensitivity, we reran the main regression analyses including both maternal anxiety symptoms (i.e., STAI) and depression symptoms (i.e., EPDS) as covariates, repeating the models for each time point that maternal mental health was measured (i.e., prenatal, 3 months, 12 months, and 30 months). Second, given prior research showing sex-specific effects of associations of maternal sensitivity with *NR3C1* 1f methylation (Conradt et al., 2019), we repeated the main regression analyses including an interaction term for maternal sensitivity at each time point by child sex in separate models. We probed significant interactions by inspecting the sex-specific slopes

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Table 1. Descriptive statistics

	N	*M*	*SD*	Min–Max
Child sex (% female)	145	47.6%		
Birthweight (g)	145	3604.10	452.66	2670–4600
Maternal age at delivery	145	32.64	3.86	21.10–42.90
Maternal sensitivity	139			
5 weeks		5.31	2.31	1.00–9.00
12 months		4.31	1.29	1.00–7.00
30 months		5.30	0.70	3.25–6.50
% Missing		4.3%		
CBCL 6 years				
Internalizing	134	4.16	3.99	0.00–21.00
Externalizing		6.44	5.29	0.00–26.00
% Missing		7.6%		
CBCL 10 years	127			
Internalizing		5.66	5.56	0.00–35.00
Externalizing		6.32	6.00	0.00–30.00
% Missing		12.4%		

Note. CBCL = Child Behavior Checklist.

and applied FDR correction to deal with multiple testing for the main effects.

Results

Preliminary analysis

Descriptive statistics for the sample characteristics, maternal sensitivity scores, and CBCL scores are shown in Table 1 and associations between these variables are reported in Table 2. Notably, across all time points, there were no significant associations of maternal sensitivity with child internalizing or externalizing behavior. However, we continued with the planned mediation analyses given that the absence of a direct association between the independent and dependent variables does not rule out the possibility of an indirect effect via a mediator (Hayes, 2013).

As for methylation levels, descriptive statistics are shown in Supplementary Table 1 and associations with the study covariates are reported in Supplementary Table 2. DNA methylation levels across the 25 CpG loci were consistent with hypomethylation of this promoter region (beta range: 0.01–0.12) with significant associations between methylation levels at several loci and the study covariates, with the exception of the two genetic PCs. Furthermore, methylation levels were significantly correlated between almost a third of CpG loci, as shown in Figure 1; these correlations were mostly positive, weak to moderate and did not show a pattern based on the spatial proximity of the loci.

Associations between maternal sensitivity and NR3C1 methylation

As shown in Table 3, maternal sensitivity at age five weeks was associated with methylation levels at two CpG loci after FDR correction for multiple testing. Specifically, higher maternal sensitivity scores were associated with significantly lower methylation levels at cg21702128 ($b = -0.002$, $p = <.001$) and cg04111177 ($b = -0.001$, $p = <.001$). In other words, each one unit increase in maternal sensitivity at age five weeks was associated with a 0.2% decrease in methylation levels at cg21702128 and a 0.1% decrease in methylation levels at cg04111177. In contrast, there were no significant associations of maternal sensitivity at either 12 months or 30 months with children's methylation levels after correcting for multiple testing. As such, in the mediation analyses we tested possible indirect effects of maternal sensitivity at five weeks on child internalizing and externalizing behavior via methylation levels at cg21702128 and cg04111177.

Indirect effects of maternal sensitivity on child behaviors via NR3C1 methylation

The results of the mediation analyses are presented in Table 4. Maternal sensitivity at five weeks did not significantly predict child internalizing or externalizing behavior at age 6 or 10 years in the total and direct models. Moreover, there were no significant indirect effects, indicating that cg21702128 and cg04111177 methylation levels did not mediate an effect of maternal sensitivity at age five weeks on child internalizing or externalizing behavior at either age 6 or 10 years. Likewise, there were no significant indirect effects when child self-reported internalizing and externalizing behavior scores at age 10 years were used in the mediation analyses instead of maternal reports.

Exploratory analyses: associations between NR3C1 methylation and child behaviors

Coefficients and uncorrected p-values for the associations of methylation levels at individual CpG loci with child internalizing and externalizing behavior at ages 6 and 10 years are shown in Table 5. Partial correlations revealed a positive association of cg01967637 methylation levels with internalizing behavior at age 6 years, a negative association of cg00629244 methylation levels with internalizing behavior at age 6 years, and a positive association of cg19135245 methylation levels with internalizing at age 10 years. However, these associations did not remain significant after correction for multiple testing. No significant associations were found between methylation levels at any of the 25 CpG loci and children's externalizing behavior at ages six or 10 years. No significant associations were found after correction for multiple testing when including child self-reported internalizing and externalizing behavior scores at age 10 years either.

Post hoc analyses

Maternal mental health

Pearson's correlations revealed one significant association between maternal sensitivity and maternal mental health: specifically, a weak negative correlation between maternal sensitivity and maternal depression scores at infant age 30 weeks ($r = .20$, $p = .006$). Furthermore, there were no meaningful changes to the results for the associations of maternal sensitivity with *NR3C1* methylation levels, or for the mediation analyses, when maternal anxiety and depression scores during pregnancy or at infant age 3 months, 12 months, or 30 months were included in the models.

Child sex

There were no moderating effects of child sex on the associations between maternal sensitivity at infant age 5 weeks or 12 months and *NR3C1* methylation. However, child sex moderated the association between maternal sensitivity at age 30 months and cg21702128 methylation levels (interaction term: $b = 0.01$,

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Table 2. Correlation coefficients for associations between study variables

	1	2	3	4	5	6	7	8	9
1. Child sex	–								
2. Birthweight[a.]	.20*								
3. Maternal age at delivery	.05	.11							
4. Maternal sensitivity 5 weeks	.01	.03	−.06						
5. Maternal sensitivity 12 months	−.16	.00	−.10	.12					
6. Maternal sensitivity 30 months	−.01	−.06	−.13	.03	.03				
7. Internalizing 6 years	.01	−.07	−.20*	−.10	.05	.04			
8. Externalizing 6 years	.03	−.13	−.08	−.13	−.08	−.06	.47**		
9. Internalizing 10 years	.06	−.12	−.04	−.06	−.15	−.04	.55**	.48**	
10. Externalizing 10 years	.06	−.08	.03	−.15	−.13	−.08	.23**	.66**	.46**

Note. Pearson's correlation coefficients are shown, except for child sex where the biserial-point correlation coefficient is reported.
[a]M_{boys} = 3689g, M_{girls} = 3510g; *p <. 05, **p < .01

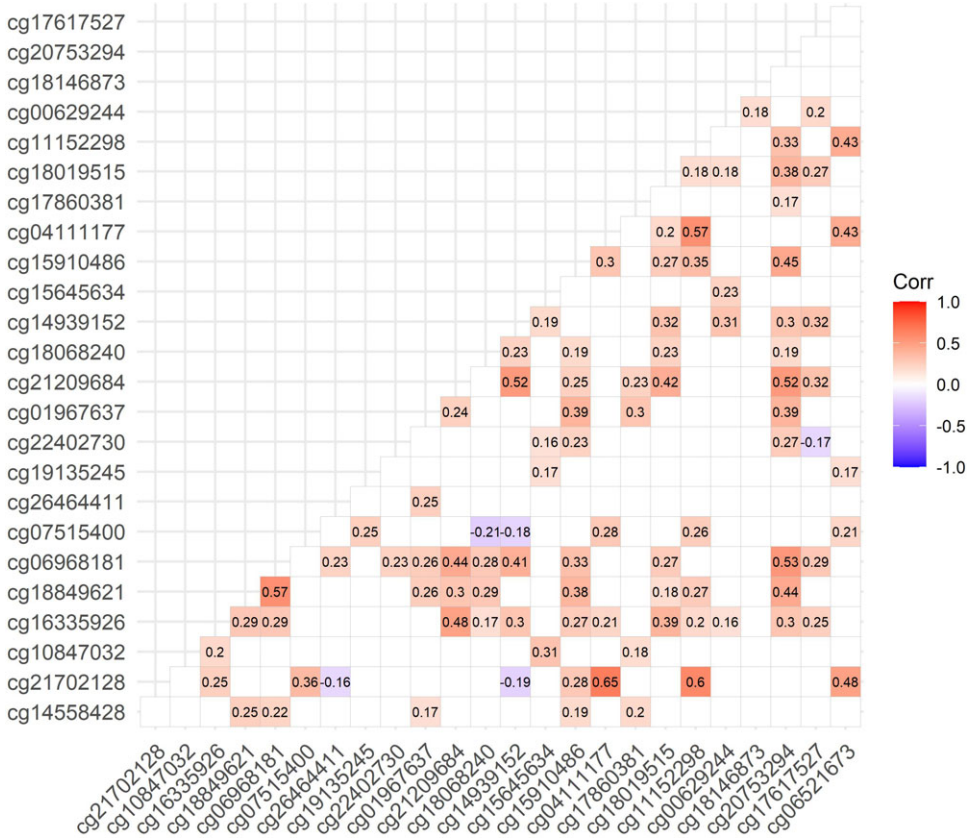

Figure 1. Heat map of significant correlations in methylation levels between 25 CpG Loci at the NR3C1 promoter region. *Note.* The figure shows the significant ($p < .05$) Pearson's correlation coefficients between methylation levels of the CpG loci at the *NR3C1* promoter. The strength of the association is visualized on a color spectrum with red representing positive correlations and blue negative correlations as indicated in the key to the right of the heat map. The heat map includes, in chromosome location order, all CpG loci at the *NR3C1* promoter region whose methylation levels are described by the EPIC array (GRCh37/hg19 chr5:142,782,071 to chr5:142,785,071).

$SE = .003$, $p = <.001$). Probing of the sex-specific slopes revealed a positive association between maternal sensitivity at 30 months and methylation levels cg21702128 in girls ($b = 0.005$, $SE = .003$, $p = .044$), whereas for boys the association was negative ($b = -0.007$, $SE = .002$, $p = .006$). However, neither association remained significant after FDR correction.

Discussion

The current study suggests that higher maternal sensitivity in the first weeks of infancy is associated with lower methylation levels at two loci at the *NR3C1* promoter region in later childhood. However, the study also highlights that the timing of caregiving quality may be particularly important given that maternal sensitivity later in infancy (i.e., 12 and 30 months) was not associated with children's *NR3C1* methylation levels. Furthermore, contrary to our hypothesis, the study showed no associations between maternal sensitivity and child internalizing and externalizing behaviors at ages 6 or 10 years – neither directly nor indirectly via *NR3C1* methylation.

In the current study, two CpG loci – cg21702128 and cg04111177 were significantly less methylated in 6-year-old children who had more sensitive mothers when they were 5 weeks old. These findings are in line with previous research that found

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Table 3. Standardized regression coefficients for associations of cpG methylation levels at age 6 with maternal sensitivity at 5 weeks, 12 months, and 30 months of age

	Maternal sensitivity					
	5 weeks		12 months		30 months	
CpG loci	*β*	*p*	*β*	*p*	*β*	*p*
cg14558428	0.04	.658	<0.01	.960	−0.05	.603
cg21702128	−0.32*	<.001	0.10	.242	0.02	.785
cg10847032	−0.06	.509	<0.01	.963	0.08	.369
cg16335926	−0.10	.253	<0.01	.960	0.02	.837
cg18849621	−0.03	.777	0.01	.951	0.02	.860
cg06968181	0.02	.814	−0.06	.515	0.06	.461
cg07515400	−0.06	.483	−0.05	.574	0.15	.084
cg26464411	0.19	.027	0.16	.060	−0.05	.515
cg19135245	0.01	.899	0.14	.128	0.06	.460
cg22402730	−0.04	.661	0.05	.566	<−0.01	.970
cg01967637	0.22	.012	−0.10	.272	0.02	.835
cg21209684	0.02	.817	−0.05	.553	0.05	.539
cg18068240	−0.12	.172	0.11	.227	−0.02	.792
cg14939152	0.04	.616	−0.09	.333	0.07	.452
cg15645634	−0.09	.290	0.04	.636	0.03	.756
cg15910486	0.08	.350	−0.02	.834	<0.01	.946
cg04111177	−0.32*	<.001	0.12	.151	0.05	.596
cg17860381	−0.01	.930	−0.04	.589	−0.04	.584
cg18019515	−0.16	.074	0.03	.694	0.03	.738
cg11152298	−0.23	.009	0.16	.074	−0.05	.562
cg00629244	−0.03	.768	0.06	.529	−0.01	.918
cg18146873	−0.02	.820	0.09	.300	0.01	.906
cg20753294	0.04	.618	0.02	.863	0.08	.373
cg17617527	−0.04	.674	−0.13	.146	−0.03	.761
cg06521673	−0.12	.170	0.01	.924	0.06	.444

Note. $N = 139$, β = standardized regression coefficients, covariates: buccal cell count, genetic PC1 and PC2, maternal age, child birthweight, child sex.
*Significant after FDR correction for multiple testing.

Table 4. Regression and bootstrapping results for testing effects of maternal sensitivity on child internalizing/externalizing behavior at age 6 and 10 as mediated by Cg21702128 and Cg04111177 methylation at age 6

	Internalizing behavior		Externalizing behavior	
	6 years	10 years	6 years	10 years
	B (SE)	*B (SE)*	*B (SE)*	*B (SE)*
Total effect models				
Maternal sensitivity at 5 weeks	−0.02 (0.01)	−0.01 (0.01)	−0.02 (0.01)	−0.02 (0.02)
Direct effect models				
Maternal sensitivity at 5 weeks	−0.02 (0.01)	−0.01 (0.01)	−0.02 (0.01)	−0.02 (0.02)
cg21702128 methylation	−3.60 (2.82)	−0.48 (2.90)	1.44 (2.77)	−0.15 (3.23)
cg04111177 methylation	3.13 (3.92)	0.61(4.09)	−5.05 (3.85)	0.52 (4.56)
Indirect effects[a]	0.02 (0.33)	<0.01 (0.04)	0.03 (0.04)	<−0.01 (0.04)
CI	−0.05, 0.09	−0.08, 0.10	−0.05, 0.11	−0.07, 0.08

Note. $N_{6\ years} = 134$, $N_{10\ years} = 127$, B = unstandardized regression coefficients, CI = 95% bias-corrected confidence interval for the significance of the indirect effects; all CIs cross zero indicating no significant effects. Covariates: maternal sensitivity at age 12 months and 30 months, buccal cell count, genetic PC1 and PC2, maternal age, child birthweight, child sex.
[a]Completely standardized indirect effects of maternal sensitivity at 5 weeks on the dependent variables.
*$p < .05$

significant associations of children's methylation levels at these two loci with severely disrupted caregiving (Weder et al., 2014). Furthermore, cg04111177 is located in the exon 1F region where methylation levels in 5-month-old infants were found to be cross-sectionally associated with maternal sensitivity in earlier studies using pyrosequencing (Conradt et al., 2016, 2019). Thus, the current study builds on previous findings by suggesting that links between maternal sensitivity and *NR3C1* methylation extend into childhood.

In relation to the functional relevance of cg21702128 and cg04111177, methylation at these sites has been linked to functioning of the HPA axis. Specifically, methylation at cg04111177 was found to predict morning cortisol values in school-aged children, although the direction of the effect was not reported (Weder et al., 2014). Meanwhile, higher methylation at cg21702128 – located in the exon 1D region – was found in patients with endogenous Cushing's syndromes, which is characterized by heightened cortisol levels (Glad et al., 2017). Therefore, differential methylation at these two CpG loci may be related to regulation of the HPA axis. Yet, as in the current study, prior research has not established a link between methylation levels at either loci and internalizing or externalizing behavior (Cicchetti & Handley, 2017; Radtke et al., 2015; Weder et al., 2014).

As for the importance of developmental timing of sensitive caregiving, we found associations between children's *NR3C1* methylation levels at age 6 years and maternal sensitivity measured at age 5 weeks, but not at ages 12 and 30 months. These findings are partly in keeping with prior research and suggest a sensitive period for *NR3C1* methylation in response to maternal sensitivity before age 12 months (Conradt et al., 2016, 2019; Dall' Aglio et al., 2020; Dunn et al., 2019). However, it should be noted that there was a difference in the measurement of maternal sensitivity at age 5 weeks versus 12 and 30 months, which matches methodological differences between previous studies in younger versus older infants. As well as the use of different observational coding systems, maternal sensitivity was observed during a mild stressor at age 5 weeks (i.e., a bathing session) compared to a play task at later ages. Earlier research has indicated that maternal sensitivity ratings can differ in situations of infant distress versus non-distress and differentially predict child outcomes (Leerkes et al., 2012). That said, both measures used in the current study have been found to detect improvements in sensitivity following a parenting intervention to increase sensitivity, which supports the likelihood that they are measuring the same construct (Mesman & Emmen, 2013).

In terms of child behavioral outcomes, maternal sensitivity did not predict levels of child internalizing or externalizing behaviors at ages 6 or 10 years in the current sample, nor did we find indirect effects via *NR3C1* promoter methylation levels. A possible reason

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Table 5. Spearman's rho coefficients and P-values for partial correlations of NR3C1 cpG loci methylation levels at age 6 with internalizing/externalizing behavior at age 6 and 10 years

	Internalizing				Externalizing			
	6 years		10 years		6 years		10 years	
CpG loci	r_s	p	r_s	p	r_s	p	r_s	p
cg14558428	.04	.614	−.12	.179	−.13	.131	−.09	.319
cg21702128	−.01	.953	.02	.851	.00	.999	.01	.911
cg10847032	.07	.405	−.02	.813	.13	.139	.10	.281
cg16335926	−.01	.918	−.02	.786	.01	.949	.00	.964
cg18849621	−.06	.477	−.03	.759	−.05	.585	−.07	.416
cg06968181	−.06	.493	.09	.341	.12	.186	.10	.289
cg07515400	−.03	.727	−.03	.729	−.08	.364	−.09	.297
cg26464411	.02	.782	−.03	.743	−.07	.434	−.10	.270
cg19135245	−.17	.051	−.19	.036	−.12	.155	−.07	.419
cg22402730	.06	.504	.12	.195	.12	.168	.13	.159
cg01967637	.23	.008	.16	.075	.08	.345	.03	.779
cg21209684	.03	.760	.11	.226	.02	.820	−.02	.816
cg18068240	.10	.239	−.04	.680	.04	.643	.00	.974
cg14939152	−.03	.719	.01	.876	.00	.971	.05	.548
cg15645634	.14	.100	.08	.357	.07	.394	.01	.880
cg15910486	.02	.795	−.06	.543	.06	.494	.03	.703
cg04111177	.05	.574	.04	.635	−.06	.520	.06	.535
cg17860381	−.01	.906	.05	.563	.09	.321	.01	.900
cg18019515	−.03	.739	.13	.163	−.04	.618	.06	.527
cg11152298	−.05	.542	−.04	.675	−.01	.874	.04	.682
cg00629244	−.18	.036	−.11	.244	−.03	.707	.00	.968
cg18146873	.02	.838	−.02	.811	.03	.737	.08	.353
cg20753294	.10	.250	.12	.189	.00	.992	−.15	.097
cg17617527	.03	.742	.03	.764	.03	.744	−.02	.790
cg06521673	.08	.340	−.01	.901	−.03	.693	−.10	.272

Note. $N_{6\ years} = 134$, $N_{10\ years} = 127$. covariates: buccal cell count, genetic PC1 and PC2, maternal age, child birthweight, and child sex.

might be the relatively long follow-up from the measurement of maternal sensitivity to child behavior, during which time other factors (e.g., parenting style, family support, and community social support) may have fostered resilience to internalizing and externalizing problems (Fritz et al., 2018). Such an explanation could also account for mixed evidence regarding the influence of maternal sensitivity on internalizing and externalizing behaviors in other community samples (Campbell et al., 2007; Kok et al., 2013; Propper et al., 2007). Moreover, most research that links higher *NR3C1* methylation to increased psychopathology does so in the context of severe adversities (Palma-Gudiel et al., 2015; Parade et al., 2016; Tyrka et al., 2016; Watkeys et al., 2018). However, the current study sample comprised healthy mother–infant dyads of mainly higher socioeconomic status who were thus less likely to have been exposed to extreme stress (Turner & Avison, 2003) and were at lower risk for psychopathology (van Oort et al., 2011). This was reflected in low *NR3C1* methylation levels and low child behavior scores across the current study sample; the lack of variation in the data hence potentially created difficulties for detecting associations. Future studies could recruit more heterogenous samples, for example including children with known risk factors for psychopathology, and consider the moderating effects of protective factors that may influence the potential pathway from caregiving to child behavior via DNA methylation. Also mentionable is that the small statistical effects of maternal sensitivity on children's methylation levels may not necessarily impact gene expression, and thus subsequent child behavior, which highlights a need for future studies that include measures of GR gene expression and HPA axis functioning to better assess the biological relevance of associations between caregiving and *NR3C1* methylation.

This study is not without limitations. For example, the study was correlational and methylation was not measured prior to exposure to caregiving, which means that we cannot conclude that higher sensitivity causes a reduction in methylation levels at the *NR3C1* promoter region. Additionally, the relatively small sample offered little power to fully evaluate the effects of possible moderators. Moreover, methylation levels were measured from buccal cells and thus the relationship with DNA methylation in brain regions implicated in stress physiology is unknown (Jones et al., 2018); although DNA methylation levels in buccal and brain

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tissues have been found to be highly correlated at *NR3C1* ($r = .92$; Braun et al., 2019). On the other hand, in terms of strengths, the longitudinal design did allow us to measure mother–infant interactions by direct observation at multiple time points rather than relying on retrospective reports, as well as to assess longer-term associations of maternal sensitivity with children's *NR3C1* methylation levels and stress-related behaviors. Furthermore, children's internalizing and externalizing behaviors at age 10 years were also measured by child self-report rather than relying solely on parental reports. The next step would be to use intervention studies whereby methylation and behavioral data are collected before and after parental sensitivity training, thus providing causal evidence for epigenetic mechanisms that link caregiving to child behavioral outcomes (Montirosso et al., 2020; Overbeek et al., 2020). Moreover, future studies could include other caregivers, such as fathers, whose sensitivity may also be important for child development and compensate when maternal sensitivity is low (Malmberg et al., 2016).

To conclude, the current study aimed to improve our knowledge of the factors and mechanisms that protect children from the development of mental health problems in the context of universal prevention. In a community sample, we showed that typical variation in maternal sensitivity at infant age 5 weeks was associated with 6-year-old children's methylation levels at *NR3C1* loci implicated in HPA axis regulation. However, the same results did not apply for maternal sensitivity measured at infant ages 12 and 30 months, suggesting a sensitive period for caregiving effects on *NR3C1* methylation within the first year of life. Moreover, we did not find evidence that differential *NR3C1* methylation mediates a pathway from sensitive caregiving to lower levels of child internalizing or externalizing behavior; this is potentially related to the influence of other protective factors and the low risk for psychopathology in our community sample. In the future, studies using parenting interventions in vulnerable (high stress) populations could expand our understanding of the biological mechanisms linking the early caregiving environment to child mental health and provide the causal evidence needed to inform universal prevention approaches.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0954579423000226

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Conflict of Interest. None.

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