The early care environment and DNA methylome variation in childhood

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Abstract

Prenatal adversity shapes child neurodevelopment and risk for later mental health problems. The quality of the early care environment can buffer some of the negative effects of prenatal adversity on child development. Retrospective studies, in adult samples, highlight epigenetic modifications as sentinel markers of the quality of the early care environment; however, comparable data from pediatric cohorts are lacking. Participants were drawn from the Maternal Adversity Vulnerability and Neurodevelopment (MAVAN) study, a longitudinal cohort with measures of infant attachment, infant development, and child mental health. Children provided buccal epithelial samples (mean age = 6.99, *SD* = 1.33 years, *n* = 226), which were used for analyses of genome-wide DNA methylation and genetic variation. We used a series of linear models to describe the association between infant attachment and (a) measures of child outcome and (b) DNA methylation across the genome. Paired genetic data was used to determine the genetic contribution to DNA methylation at attachment-associated sites. Infant attachment style was associated with infant cognitive development (Mental Development Index) and behavior (Behavior Rating Scale) assessed with the Bayley Scales of Infant Development at 36 months. Infant attachment style moderated the effects of prenatal adversity on Behavior Rating Scale scores at 36 months. Infant attachment was also significantly associated with a principal component that accounted for 11.9% of the variation in genome-wide DNA methylation. These effects were most apparent when comparing children with a secure versus a disorganized attachment style and most pronounced in females. The availability of paired genetic data revealed that DNA methylation at approximately half of all infant attachment-associated sites was best explained by considering both infant attachment and child genetic variation. This study provides further evidence that infant attachment can buffer some of the negative effects of early adversity on measures of infant behavior. We also highlight the interplay between infant attachment and child genotype in shaping variation in DNA methylation. Such findings provide preliminary evidence for a molecular signature of infant attachment and may help inform attachment-focused early intervention programs.

Studies of maternal mental health (O'Donnell, Glover, Barker, & O'Connor, 2014; O'Donnell & Meaney, 2016; Pawlby, Hay, Sharp, Waters, & O'Keane, 2009), institutional rearing (McLaughlin et al., 2014; Nelson et al., 2007; Rutter, Kumsta, Schlotz, & Sonuga-Barke, 2012; Sonuga-Barke et al., 2017), and childhood abuse (Klengel et al., 2013; Nanni, Uher, & Danese, 2012; Spatz Widom, DuMont, & Czaja, 2007) reflect

the profound influence of the early environment on child development and later mental health. However, not all children are affected equally, with a subset of children seemingly resilient to some of the negative consequences of early life adversity. Early intervention programs that seek to mitigate the negative sequelae associated with early life adversity would benefit from a better understanding of such resilience.

The genome is one source of individual differences in sensitivity to the early environment. The negative effects of maternal antenatal anxiety on child emotional problems (O'Donnell, Glover, Holbrook, & O'Connor, 2014), working memory, and symptoms of ADHD (O'Donnell et al., 2017) are moderated, in part, by genetic variation in the child. Likewise, polygenic variation across the serotonin and dopamine signaling pathways moderates the impact of prenatal adversity (Silveira et al., 2017) and early caregiving (Belsky & van IJzendoorn, 2017; Stocker et al., 2017) on child neurodevelopment and long-term mental health outcomes.

The quality of the early caregiving environment can also contribute to individual differences in susceptibility to the negative effects of early adversity. Bergman, Sarkar, Glover, and O'Connor (2010) highlight infant attachment security as a moderator of prenatal adversity effects on infant cognitive development. In their study of women undergoing amniocentesis, the authors report an inverse association between fetal cortisol exposure and measures of cognitive development at 17 months. This relationship was only evident in infants with an insecure attachment style. Likewise, McGoron et al. (2012) report that infant attachment security limited the impact of institutional rearing on measures of child psychopathology. Taken together, these findings suggest the early caregiving environment can buffer some of the negative consequences of adversity experienced in the prenatal and early postnatal environment. The biological basis of such effects is not well understood.

Chemically stable, epigenetic modifications, such as DNA methylation, have emerged as a putative mechanism for the enduring effects of the early environment on neural development and function (Meaney & Ferguson-Smith, 2010). DNA methylation can modulate gene expression as well as splicing events (Maunakea, Chepelev, Cui, & Zhao, 2013; Maunakea et al., 2010), thus creating a diversity of transcriptional programs from a single genome. The human DNA methylome is dynamic across development (Teh et al., 2014; Ziller et al., 2013) and is altered by early social experience (Klengel et al., 2013; Lam et al., 2012; O'Donnell et al., 2018). Studies using rodent models reveal effects of parent–offspring interactions on the methylation status of a range of genes implicated in hypothalamic–pituitary–adrenal stress responses as well as synaptic function (Bagot et al., 2012; Klengel et al., 2013; Lam et al., 2012; Murgatroyd et al., 2009; O'Donnell et al., 2018; Roth, Lubin, Funk, & Sweatt, 2009; Weaver et al., 2004; Wu, Patchev, Daniel, Almeida, & Spengler, 2014; Zhang et al., 2010).

Genome-wide analyses of DNA methylation in humans reveal broad effects of childhood abuse (Mehta et al., 2013; O'Donnell et al., 2018; Suderman et al., 2012). However, few studies examine whether variations in parent–offspring interactions lying within the normal range are a source of variation in the DNA methylome. One recent study reports an association between one specific component of caregiving, the amount of physical contact between mothers and their infants, and variation in DNA methylation (Moore et al., 2017). However, Moore et al. did not examine how the *quality* of such mother–infant interactions predicted variation in the DNA methylome.

Infant attachment is an objective measure of the early care environment that associates with child mental health outcomes (Fearon, Bakermans-Kranenburg, van IJzendoorn, Lapsley, & Roisman, 2010; Groh et al., 2014; Groh, Roisman, van IJzendoorn, Bakermans-Kranenburg, & Fearon, 2012) and may buffer the effects of prenatal adversity on measures of child development (Bergman et al., 2010; McGoron et al., 2012). In this study we use a prospective, longitudinal cohort of typically developing children to describe the association between infant attachment and variation in DNA methylation across the genome.

Method and Materials

Participants

Participants ($N = 226$: 111 female, 115 male, mean age $= 6.99$, $SD = 1.33$ years) were drawn from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) cohort (O'Donnell, Gaudreau, et al., 2014). Full ethical approval for this study was provided by the institutional review boards at the Douglas Research Institute and McMaster University.

The early care environment

Infant attachment was assessed at 36 months using a modified version of the Strange Situation (Moss, Bureau, Cyr, Mongeau, & St.-Laurent, 2004). This 20-min observational assessment consists of a sequence of separations/reunions lasting 5 min each: (1) separation between mother and child; (2) reunion; (3) second separation; and (4) second reunion. Behavior was coded during each reunion episode giving rise to one of four attachment classifications: secure, avoidant, ambivalent, or disorganized (Moss, Cyr, Bureau, Tarabulsy, & Dubois-Comtois, 2005). A secure attachment style is characterized by increased exploration in the presence of the primary caregiver and generally associates with more favorable developmental outcomes. Insecure attachment is characterized by reduced exploration, even in the presence of the primary caregiver. Insecurely attached infants are further subdivided as either "avoidant" of the primary caregiver during stress or striving to maintain close proximity to the primary caregiver, but not soothed during distress (ambivalent). Disorganized attachment describes infants who fail to show an "organized" strategy for eliciting the attention of the primary caregiver during stress, who display stress-associated behaviors (e.g., hunched shoulders or freezing) and may show evidence of role-reversed behavior (e.g., attempting to control the caregiver; Moss et al., 2005). Disorganized attachment is also associated with an increased risk of later psychopathology (Fearon et al., 2010; Groh et al., 2012; Scholtens, Rydell, Bohlin, & Thorell, 2014). Trained observers scored all videos and demonstrated excellent interrater reliability (κ = 0.83). Due to the small number of avoidant ($n = 13$) and ambivalent ($n = 25$) cases, both groups were combined to form an organized–insecure category.

Biological sample collection

We collected buccal epithelial cells (Catch-All Swabs, Epicentre, USA) from all children (mean age $= 6.99$, $SD = 1.33$ years). Buccal cell collection is minimally invasive, provides a relatively homogenous cell type (squamous epithelium) for DNA methylation analyses (Berko et al., 2014;

Lowe et al., 2013), and may serve as an appropriate surrogate for neural tissue given their shared ectodermal lineage. Papavassiliou et al. (2009) report that trisomy mosaicism in buccal cells is a stronger predictor of cognitive impairment in Down syndrome patients than mosaicism determined in lymphocytes (Papavassiliou et al., 2009). Likewise, Smith et al. (2015) report greater similarity between DNA methylome profiles derived from neural tissue and saliva than blood-based data. Smith et al. suggest that this brain-saliva similarity is driven, in part, by the proportion of epithelial cells within saliva samples.

DNA methylation analyses

Genomic DNA was extracted using the Masterpure system (Epicentre, USA) and quantified using a NanoPhotometer P300 (Implen, Germany). The genomic DNA (750 ng) was bisulfite converted using the EZ-DNA Methylation Kit (Zymo Research, USA) and isothermally amplified at 37 °C for 22 hr, enzymatically fragmented, purified, and hybridized on Infinium HumanMethylation450 beadchip array (450K, Illumina). The 450K array permits quantification of DNA methylation with single-base resolution across multiple probes, including 482,421 CpG sites and 3,091 non-CpG sites (Bibikova et al., 2011). Our studies with this technology suggest strong reproducibility and good correlation with bisulfite sequencing, including reduced representational bisulfite sequencing and confirmatory pyrosequencing (Marr et al., 2014; O'Donnell et al., 2018; Pan et al., 2012). The 450K chips were scanned using the Illumina HiScan system and the image data were processed in R (R Core Team, 2014) using the Minfi package (Aryee et al., 2014).

The experiment was run in 4 batches together with a series of internal controls distributed across each batch and longitudinal samples (Forest et al., 2018). We report findings from DNA methylation data generated from the first biological sample collection, which children provided at age 6.99 years (*SD* = 1.33).

450K data processing. DNA methylation data were processed in R using the Minfi package (Aryee et al., 2014). Samples that failed standard Minfi quality control (QC; QC threshold = 10.5) were removed (Aryee et al., 2014). All remaining samples had a high call rate (>95%). Probes with a low call rate (<75%), a high detection p value ($p > .05$; Lehne et al., 2015), and a low number of beads (less than 3 in >5% of the cohort) were also removed. Predicted sex (from DNA methylation of the sex chromosomes; Aryee et al., 2014) and reported sex for all participants was consistent for all samples. Nonspecific probes or probes with single nucleotide polymorphism (SNP)-disrupting polymorphisms were removed (Chen et al., 2013; Price et al., 2013), along with all the probes on the sex chromosomes. Unwanted technical variation was accounted for, in part, using functional normalization based on principal component analysis of control probes on the 450K (Fortin et al., 2014). We also used

ComBat (Johnson, Li, & Rabinovic, 2007) to iteratively adjust our data for unwanted technical variation associated with experimental batch, array row (sentrix row), and plate position (sample column). Hierarchical clustering showed that technical replicates were more closely related to each other than to other individuals. Therefore, replicates from the same individual were combined such that each participant contributed one unique data point in our analyses. The data set was further reduced to probes showing interindividual variation, defined as probes with a range of methylation (β value ≥ 0.10) corresponding to at least a 10% difference in DNA methylation across participants. A total of 252,439 probes remained and were denoted as variably methylated probes (VMPs).

Cell type adjustments. We used the method described by Smith et al. (2015) to predict buccal epithelial cell content of each sample. The proportion of buccal cells per sample was added as a covariate to all models describing variation in DNA methylation.

Genetic analyses

We used the PsychChip and the commercially available PsychArray (Illumina) to describe genetic variation within this cohort. Only shared SNPs (between the PsychChip and the PsychArray) were considered. SNPs with a low call rate (i.e., called in <95% of samples) and samples with a low call rate (i.e., <95% of SNPs called within a sample) were removed, resulting in 223 participants and 228,562 autosomal SNPs. Next, we used the Sanger Imputation Service to impute missing genotypes, resulting in ~17M SNPs per participant.

Population structure. We filtered the genetic data set to retain SNPs in low linkage disequilibrium ($r^2 < .25$). These SNPs were then subjected to principal component analysis (PCA) in EIGENSTRAT, an established method to describe population structure (Price et al., 2006). Two principal component scores were included in all analyses to account for population stratification/ancestry effects.

Child outcome measures. We used the Bayley Scales of Infant Development II (BSID) to assess child motor development (Psychomotor Development Index; PDI) and mental development (Mental Development Index; MDI) at 36 months (Bayley, 1993). We also rated child behavior (Behavior Rating Scale; BRS) during the BSID across multiple domains (e.g., attention/arousal, orientation/engagement, emotional regulation, and motor quality) to index child performance during this mildly stressful, novel task.

Mothers completed a series of questionnaires concurrent with buccal sample collection from their children (see Table 1 for maternal and child age at time of collection). We used the maternal report from the Strengths and Difficulties Questionnaire (SDQ; Goodman, Meltzer, & Bailey, 1998) to describe

Table 1. *MAVAN 450K cohort characteristics*

MAVAN 450K Cohort	$N = 226$
Age (*SD*)	6.99 (1.33)
Biological sex	
Female (%)	111 (49.1)
Infant attachment at 36 months (%)	195
Secure	110 (48.7)
Insecure[†]	38 (16.8)
Disorganized	47 (20.8)
Missing	*31 (13.7)*
BSID at 36 months	
Behavior Rating Scale (*SD*)	122.85 (6.43)
Missing (%)	*13 (5.8)*
Mental Development Index (*SD*)	98.72 (10.86)
Missing (%)	*17 (7.5)*
Psychomotor Development Index (SD)	101.15 (12.86)
Missing (%)	*15 (6.6)*
SDQ at time of biosample collection	
Total SDQ Score (*SD*)	8.29 (4.94)
Missing (%)	*4 (1.8)*
Genetic variation/SNP data	
Available (%)	223 (98.7)
Prenatal Adversity Score (%)	180
0	63 (27.9)
1	48 (21.2)
2	46 (20.4)
3	10 (4.4)
4	9 (4.0)
≥ 5	4 (1.7)
Missing	*46 (20.4)*
Maternal age (*SD*)	
At birth of study child	30.53 (4.95)
At time of recruitment/	37.58 (4.89)
data collection for 450K cohort	
Maternal education	
University educated (% Yes)	119 (52.7)
Maternal anxiety	
STAI (*SD*)	35.55 (11.04)
Missing (%)	*5 (2.2)*

Note: BSID, Bayley Scales of Infant Development. SDQ, Strengths and Difficulties Questionnaire. STAI, Spielberger Trait Anxiety Inventory. [†]Avoidant ($n = 13$) and ambivalent ($n = 25$) attachment classifications were combined to form an "organized" insecure group.

symptoms of child emotional/behavioral problems. This well-validated screening assessment provides symptom data on four domains related to emotional difficulties, inattention/hyperactivity, conduct problems, and peer relations. A summary score of these four problem subscales contributes to the total SDQ score, which shows good predictive validity for child mental health problems (Goodman, Ford, Simmons, Gatward, & Meltzer, 2000; Goodman, Renfrew, & Mullick, 2000). Mothers also completed the Spielberger State/Trait Anxiety Inventory (Spielberger, Gorusch, & Lushene, 1970), which we used to index maternal trait anxiety at the time of biological sample collection from their child. Finally, maternal education (university educated or not) was coded as a binary variable and considered in all analyses.

Data analysis

Variably methylated probes (VMPs; $n = 252{,}439$) were subjected to PCA in R. We focus our analysis on the first 10 principal components (PCs) that account for 41.9% of the variance in our DNA methylation data (Figure 1). We used one-way analysis of variance (ANOVA) models to describe associations between infant attachment and PCs scores. ANOVA models were adjusted for measures of cellular heterogeneity in buccal samples, child biological sex and age, measures of population stratification derived from genetic data, and maternal anxiety and level of education. Attachment style was coded as a categorical nonordinal measure.

The following linear regression model was tested to identify VMPs for which methylation levels were predicted by attachment:

$$\text{Model } 1 (E\ Model): VMP \sim attachment + age + \\ sex + pop.\ strat. + BCC + mat.\ anx. + mat.\ edu,$$

where VMP represents %methylation at a single variably methylated probe; attachment is attachment style, coded into three groups: secure, insecure, and disorganized with secure as the reference category; sex is biological sex of participant, age is age in days at time of buccal sample collection; pop. strat. represents principal components one and two from a PCA of genetic data; BCC is buccal cell composition expressed as a percentage; mat. anx. represents maternal Spielberger State/Trait Anxiety Inventory score at time of biosample collection; and mat. educ. is maternal level of education as a proxy for socioeconomic status.

VMPs that were associated with infant attachment ($p < .05$) were mapped to genes using Illumina annotation and entered into a Genego Metacore analysis to identify enrichment of biological pathways and processes within our methylation data set. We used MetaCore + MetaDrug version 6.18 (Thomson Reuters, USA).

Genetic contribution to DNA methylation at attachment-associated VMPs. We sought to determine if genetic variation in *cis* (within 10 kilobases; 10 kb) contributed to variation in DNA methylation at attachment-associated VMPs. We tested for main effects of all SNPs in *cis* adjusting for relevant covariates (see Model 2). One SNP was tested per model and adjusted for relevant covariates:

$$\text{Model } 2 (G + E\ Model): VMP \sim attachment + \\ SNP_{i...j} + age + sex + pop.\ strat. + BCC + \\ mat.\ anx. + mat.\ edu,$$

where $SNP_{i...j}$ represents the first to the last SNP within a 10-kb window from the attachment-associated VMP.

In parallel, we ran a series of linear models that included an interaction term between infant attachment and each SNP in *cis* to predict DNA methylation at attachment-associated
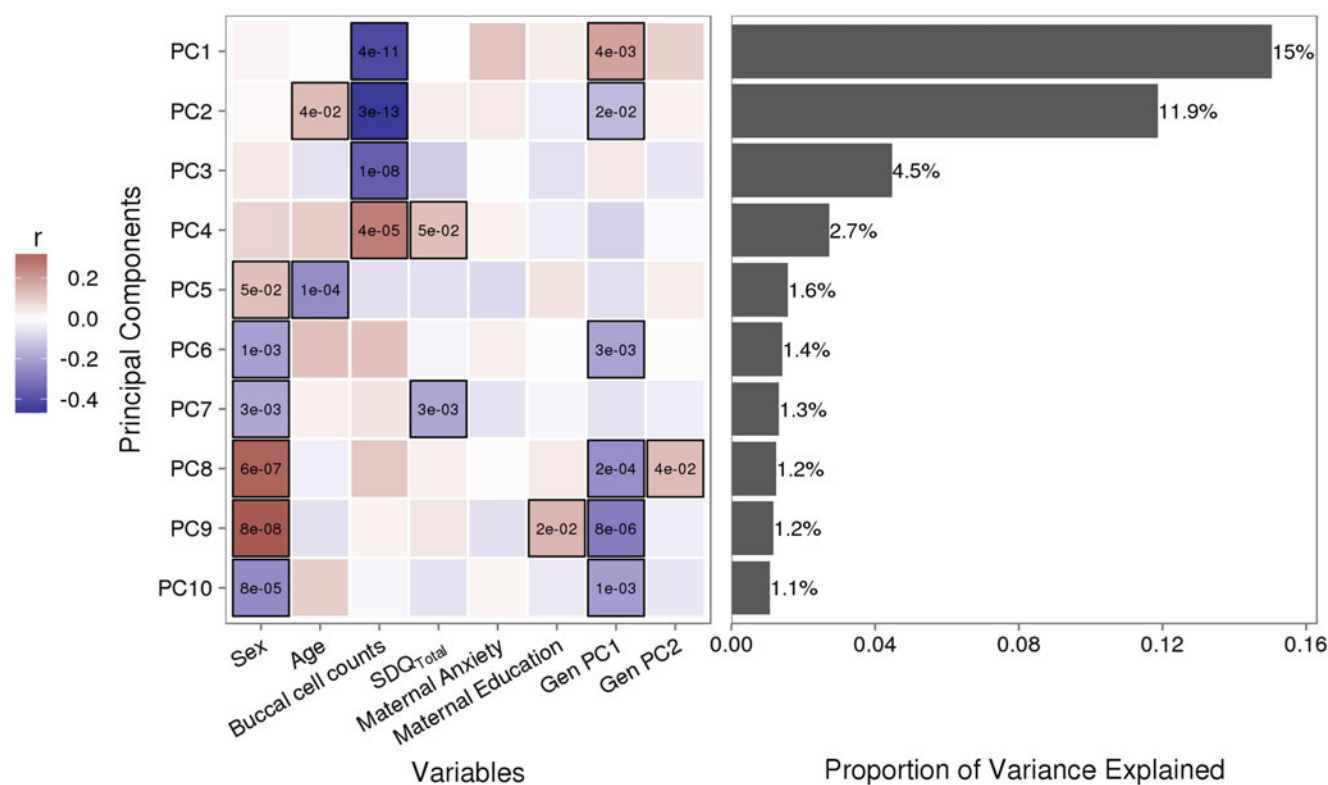
Figure 1. Sources of variation in the DNA methylome. (a) Heatmap describes the bivariate association between variables of interest and the first 10 principal components (PCs) from a principal component analysis of variably methylated probes. The *p* values are provided for significant associations (*p* < .05). (b) Bars describe the proportion of variance explained by each principal component. SDQ$_{Total}$, Strengths and Difficulties Questionnaire total scale score. GenPC1/GenPC2, first and second principal component score from principal component analysis of genetic variation.

VMPs (Model 3). One SNP and its corresponding interaction term with infant attachment was tested per model and adjusted for relevant covariates:

$$Model\ 3(G \times E\ Model): VMP \sim attachment +$$
$$SNP_{i...j} + attachment^*SNP_{i...j} + age + sex +$$
$$pop.\ strat. + BCC + mat.\ anx. + mat.\ edu,$$

where *attachment* * $SNP_{i...j}$ is the interaction term between infant attachment and the first to the last SNP within a 10-kb window from the attachment-associated VMP.

We ranked these additive (G + E) models per VMP to identify the SNP in *cis* that accounted for the largest proportion of variance in DNA methylation (i.e., highest model adjusted R^2) at a given VMP. Next, we ranked all interaction (G x E) models per VMP to identify the SNP, which in interaction with infant attachment, accounted for the largest proportion of variance in DNA methylation at a given VMP (i.e., highest model adjusted R^2). We selected VMPs for which the top ranked SNP from the additive and interaction model was the same, permitting a direct comparison of models (i.e., a comparison of nested models; Belsky, Pluess, & Widaman, 2013). We compared adjusted R^2 across each of these three models to identify the model that best explained DNA methylation at attachment-associated VMPs (i.e., the model

with the highest adjusted R^2). We also compared our results based on percentage variance explained (adjusted R^2) to a parallel analysis using the Akaike information criterion (AIC), which permits the comparison of nonnested models.

Supplementary analyses

The MAVAN study collected data on a number of measures related to the prenatal period. We used these data to determine if infant attachment buffers the negative effects of prenatal adversity on infant (BSID) and child (SDQ) outcomes, in line with previous reports for different cohort studies (Bergman et al., 2010; McGoron et al., 2012).

Prenatal adversity. We used a cumulative measure of prenatal adversity described and validated previously within the MAVAN cohort (Silveira et al., 2017). Information on maternal antenatal illness, birth size, gestational age, household income, lack of material resources, domestic violence, marital strain, maternal anxiety, and maternal depression was combined to form a cumulative score that ranged from 0 to 9. Data on prenatal adversity were available on *n* = 180 participants included in the subsample of the MAVAN cohort (see Table 1). We used linear regression analysis to test the association between prenatal adversity and (a) infant BSID and
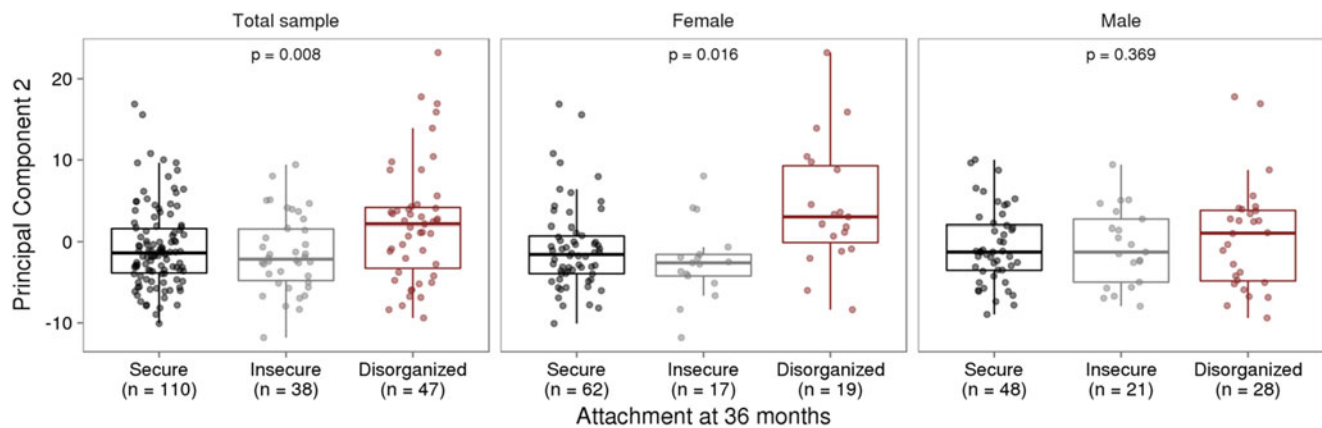
Figure 2. Infant attachment and variation in DNA methylation. (a) Infant attachment associates with principal component two (PC2). PC2 accounts for 11.9% of the variance in DNA methylation of variably methylated probes. (b) Sex-stratified analyses show these effects are evident in females but not in males. The *p* values are derived from analysis of variance.

(b) child SDQ scores. We also included interaction terms between prenatal adversity and infant attachment classification.

Biological sex-specific effects. We also carried out a series of analyses stratified by biological sex to examine differences in the association between infant attachment and variation in DNA methylation between males and females.

Results

Table 1 describes the characteristics of the children and mothers included in our analysis. There were no significant differences in the age ($p = .081$) or biological sex ($p = .198$) of participants across the different attachment classifications.

Infant attachment and child outcomes

One-way ANOVA models showed a significant association between infant attachment and the Mental Development Index, MDI: $F (2, 189) = 13.864$, $p < .001$, Cohen's $f = .39$, and Behavior Rating Scale, BRS: $F (2, 192) = 10.74$, $p < .001$, Cohen's $f = 0.34$, from the BSID at 36 months. Infant attachment was associated with infant psychomotor development (PDI) at trend level, $F (2, 190) = 2.385$, $p = .095$, Cohen's $f = 0.16$. Posthoc analyses of these significant associations revealed differences between the secure and disorganized groups on BRS and MDI scores with large effect sizes (Cohen's $d \geq 0.84$), and between the insecure and disorganized groups with medium effect size (Cohen's $d \geq 0.46$; BRS: all adjusted $p \leq .026$; MDI: all adjusted $p \leq .001$). No significant differences were observed between infants with a secure and those with an insecure attachment classification for MDI or BRS scores. These results were largely unchanged following adjustment for child biological sex, population stratification, and maternal education, MDI: $F (2, 187) = 12.425$, $p < .001$; BRS: $F (2, 190) = 8.679$, $p < .001$; PDI: $p > .1$.

One-way ANOVA models showed a significant association between infant attachment and child mental health, total

SDQ: $F (2, 192) = 3.389$, $p = .036$, Cohen's $f = 0.19$). Post hoc tests revealed significant differences between the secure and disorganized groups (adjusted $p = .033$) with a medium effect size (Cohen's $d = 0.45$), with no difference between secure and insecure groups. This association was somewhat reduced following adjustment for child age, biological sex, population stratification, and maternal education, total SDQ: $F (2, 190) = 2.654$, $p = .073$. However, when we considered maternal anxiety at the time of SDQ report, to control for potential reporter bias, infant attachment was no longer associated with child total SDQ score ($p = .513$).

Infant attachment and variation in DNA methylation: Principal components analysis

Figure 1 illustrates the bivariate associations between predictors of interest and the first 10 PC scores from a PCA of VMPs.

Infant attachment was significantly associated with PC2, $F (2, 189) = 4.964$, $p = .008$, Cohen's $f = 0.24$, after adjustment for child age, biological sex, cellular heterogeneity within buccal samples, population stratification, and measures of maternal education and anxiety at time of biosample collection (Figure 2).

Infant attachment and variation in DNA methylation: VMP analysis

No individual VMP passed a Benjamini–Hochberg false discovery rate correction. However, the *p* value distribution for the model was substantially skewed (Figure 3). A large number of VMPs were associated with infant attachment at a nominal level (unadjusted $p = .05$). This skewed *p* value distribution differs from the uniform flat distribution expected under the null hypothesis (i.e., no association between DNA methylation and infant attachment). These differences were most striking for comparisons between secure and disorganized groups ($n = 35,761$, unadjusted $p < .05$).
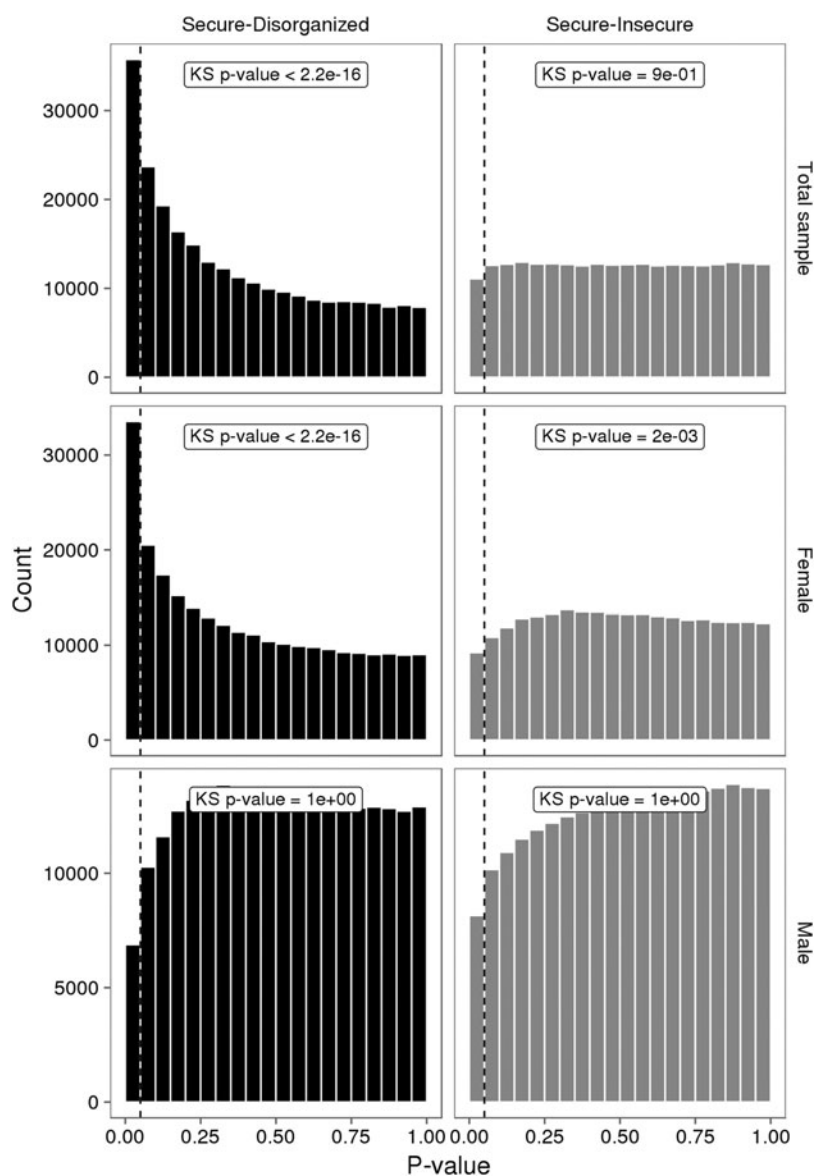
Figure 3. The *p* value distributions for linear models describing the association between infant attachment and variably methylated probes. The *p* values derived from one-tailed Kolmogorov–Smirnov (KS) test for uniformity of distribution.

Of note, attachment-associated VMPs (secure vs. disorganized $n = 35,761$, nominal $p < .05$) were located in genes for which gene expression has been shown to covary with individual differences in early life experience (Anacker et al., 2013; Klengel et al., 2013; Pena, Neugut, & Champagne, 2013; Wu et al., 2014). These included multiple VMPs within the glucocorticoid receptor (*NR3C1*; cg08845721, cg13648501, cg16586394, cg19457823, cg20753294), estrogen receptor-alpha (*ESR1*; cg07455133, cg07746998, cg08907436, cg17264271, cg25490334), as well as genes involved in glucocorticoid signaling *(SGK1:* cg06849960, cg09872934, *cg11856561 cg12871835 SGK2:* cg21685427, *FKBP5:* cg07061368, cg07843056 and *POMC*: cg11894631; see online-only Supplemental Table S.1 for all attachment-associated VMPs).

Pathway analysis of attachment-associated VMPs (nominal $p < .05$) showed significant enrichment for multiple pathways associated with cell signaling and cell adhesion (Table 2), including the Ephrin signaling pathway.

Child genetic variation contributes to DNA methylation at attachment-associated VMPs

We focused our genetic analysis on attachment-associated VMPs identified from our comparison of secure versus disorganized groups (see Model 1) with at least one SNP in *cis* ($n = 30,965$). We identified the SNP from the additive (G + E model; Model 2) or interaction (G x E model; Model 3) models that accounted for the largest proportion of variance in DNA methylation at a specific VMP (see Methods).

Table 2. *Pathway and process enrichment analysis of attachment-associated variably methylated probes*

Rank	Biological pathways	*p* value	FDR
1	Ephrin signaling	2.57E-05	2.45E-02
2	POMC processing	5.91E-05	2.68E-02
3	VEGF signaling via VEGFR2	8.46E-05	2.68E-02
4	Receptor-mediated axon growth repulsion	1.20E-04	2.86E-02
5	SDF-1 signaling in hematopoietic stem cells	3.51E-04	4.81E-02
	Biological processes		
1	Cell adhesion: Attractive and repulsive receptors	1.58E-05	1.48E-03
2	Cell adhesion: Cell junctions	2.14E-05	1.48E-03
3	Potassium transport	2.79E-05	1.48E-03
4	Cell adhesion: Platelet aggregation	4.10E-05	1.63E-03
5	Neurogenesis: Axonal guidance	7.38E-05	2.35E-03

Notes: Unadjusted *p* values and *p* values adjusted for multiple comparisons using the false discovery rate (FDR) are provided.

For 18,042 of these attachment-associated VMPs the same SNP emerged from the additive (Model 2) and interaction model (Model 3). These nested models permitted a direct comparison of adjusted R^2 across models. We observed that the interaction (G x E) model explained the largest proportion of variance (highest adjusted R^2) in DNA methylation for 58.9% of attachment-associated VMPs (see Figure 4). In total, 76.4% of these attachment-associated VMPs benefited from the inclusion of a measure of child genetic variation (Figure 4). We performed a parallel analysis and used AIC to index model fit for prediction models that considered different SNPs across additive and interaction models (i.e., nonnested models) in the prediction of DNA methylation at all attachment-associated VMPs ($n = 30,965$). These analyses revealed somewhat similar results; model fit for approximately half (48.8%) of all attachment-associated VMPs was improved (i.e., had a lower AIC) when a measure of child genetic variation was considered.

Supplementary analyses

Infant attachment and moderation of prenatal adversity effects on child outcomes. Measures of prenatal adversity described previously (Silveira et al., 2017) and child outcomes were available on a subset of this sample. Linear regression models revealed that prenatal adversity predicted BRS ($B = -0.934$, $p = .018$) scores from the BSID at 36 months independent of infant attachment and all covariates. Prenatal adversity was associated with both infant MDI ($p = .079$) and PDI scores ($p = .10$) at trend level. We observed a significant interaction between prenatal adversity and attachment security to predict the BRS score, $F(2, 153) = 5.82$, $p = .004$, with an effect of prenatal adversity on infant BRS in the disorganized group only ($B = -3.14$, $p < .001$). A similar pattern was observed for MDI scores but did not reach statistical significance, $F(2, 151) = 2.39$, $p = .095$ (see Figure 5). Prenatal adversity did not interact with infant attachment to predict infant PDI ($p > .1$).

Prenatal adversity predicted child total SDQ score ($B = 0.71$, $p = .028$) independent of all covariates considered in our SDQ model, including measures of infant attachment and maternal trait anxiety (see Methods). However, prenatal adversity did not interact with infant attachment to predict child total SDQ scores. (See Supplemental Table S.2 for all prenatal adversity linear regression results).

Sex-specific effects of infant attachment on DNA methylation of attachment-associated VMPs. We carried out exploratory analyses to examine potential sex differences in the association between infant attachment and variation in DNA methylation. Analyses stratified by biological sex of our PCA of VMPs revealed a significant association between PC2 and infant attachment in females but not in males (Figure 2). Similar results were observed from our analyses of infant attachment and DNA methylation at individual VMPs. Differences in DNA methylation between secure and disorganized classifications were most pronounced in females but not in males (see Figure 3).

Discussion

We describe the association between infant attachment at 36 months of age and DNA methylome variation in childhood. To our knowledge this is the first and largest assessment of infant attachment and variation in DNA methylation carried out to date. These findings contribute to a growing literature describing the biological embedding of early experience and suggest a persisting influence of infant attachment on variation in DNA methylation. Our study also provides the first step toward characterizing a molecular signature of infant attachment, which may inform early interventions that seek to promote attachment security.

Our PCA of VMPs showed that infant attachment associates with a significant portion of the variance across the DNA methylome. These data extend those of Moore et al. (2017),
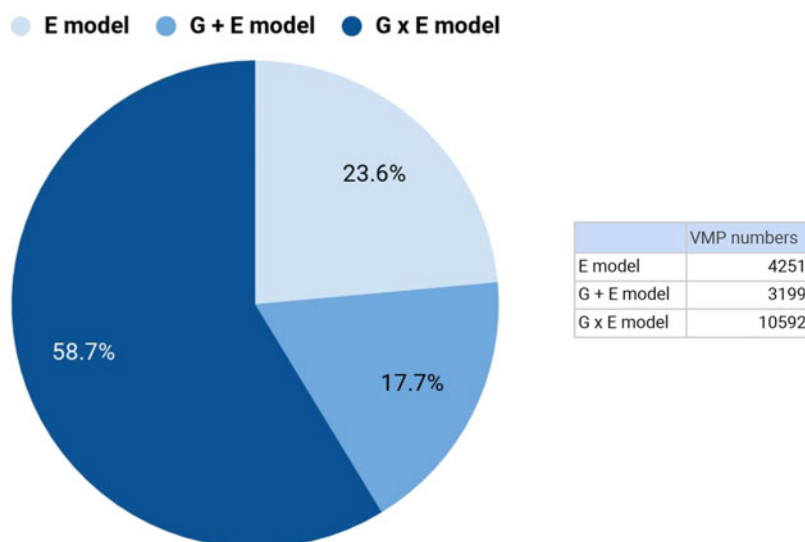
Figure 4. Genetic variation contributes to DNA methylation of attachment-associated variably methylated probes (VMPs). DNA methylation at VMPs were described using linear models that included infant attachment as the environmental predictor (E Model: light blue), the additive effects of infant attachment and a single nucleotide polymorphism (SNP) within 10kb from a VMP (G + E Model: blue), and an interaction model (G×E Model: dark blue) that included infant attachment, a SNP within 10kb from a VMP, and the interaction term between this SNP and infant attachment style. The proportion of variance explained (adjusted R^2) was used to compare models. Percent values on chart denote the number of VMPs that are best explained (highest adjusted R^2) by a specific model, and corresponding numbers of VMPs are provided (see insert). All models were adjusted for buccal cell proportions, population stratification, child age and biological sex, maternal education, and anxiety. kb, kilobase.

and suggest normal variations in the quality of the early caregiving environment may influence variation across the DNA methylome.

Across both our pathway and process enrichment analyses, we observed enrichment for genes related to cell adhesion/cell migration, such as the Ephrin signaling pathway. Ephrins, which bind tyrosine kinase Eph receptors, are critically involved in neuronal interaction, cell migration, and cell differentiation. This is the first report in humans documenting an association of the early care environment and DNA methylation of Ephrin family members; however, our findings draw support from a number of preclinical models. For example, Provençal et al. (2012) report differential DNA methylation of Ephrin family members (ENFB2/ENFA5) in T cells and prefrontal cortex samples from nonhuman primates exposed to their peer-rearing model of early adversity (Provençal et al., 2012). Similarly, female Ephrin knock-out mice (Ephrin-A5$^{-/-}$) exhibit reduced levels of maternal care toward their offspring (Sheleg et al., 2017). Together with our own data, these findings serve to highlight Ephrin signaling as a novel pathway both sensitive to the effects of the early care environment, and potentially, for promoting caregiving behavior.

We also observed significant enrichment for POMC processing within attachment-associated VMPs. However, we note that this enrichment derives in part from the large number of protein products, which arise from the cleavage of POMC (Pritchard & White, 2007; Wu et al., 2014). The methylation of the *Pomc* promoter is influenced by variation in maternal care (Wu et al., 2014), and POMC is centrally

involved in glucocorticoid signaling. We also observed differential methylation for a number of VMPs within the glucocorticoid receptor *NR3C1* and glucocorticoid-sensitive targets, including *FKBP5* and *SGK1,* across attachment groups. *SGK1* gene expression is increased in blood samples from depressed individuals and also within the hippocampus of prenatally stressed rats (Anacker et al., 2013). Klengel et al. (2013) report altered *FKBP5* methylation and glucocorticoid sensitivity following exposure to childhood trauma that covaries with hippocampal volume as well as with clinical symptoms and treatment outcomes in posttraumatic stress disorder (Yehuda et al., 2013).

We also found that infant attachment was associated with variation in DNA methylation at multiple sites within *ESR1,* which encodes estrogen receptor alpha (ERα). Unfortunately, we were unable to quantify the effects of infant attachment and associated differential methylation of *ESR1* on its gene expression. Nevertheless, the variation in *ESR1* methylation as a function of attachment is consistent with studies showing that the frequency of pup licking/grooming in the rat predicts DNA methylation of an *Esr1* promoter and *Esr1* expression in the hypothalamus and amygdala (Champagne & Meaney, 2006; Pena et al., 2013). Binding of ERα to its consensus binding site can influence DNA methylation of target genes (Guintivano, Arad, Gould, Payne, & Kaminsky, 2013; Kangaspeska et al., 2008; Metivier et al., 2008) consistent with transcription factor-mediated remodeling of the DNA methylome (Stadler et al., 2011; Weaver et al., 2007).

The association between infant attachment and methylation of *ESR1* is of interest considering the known association
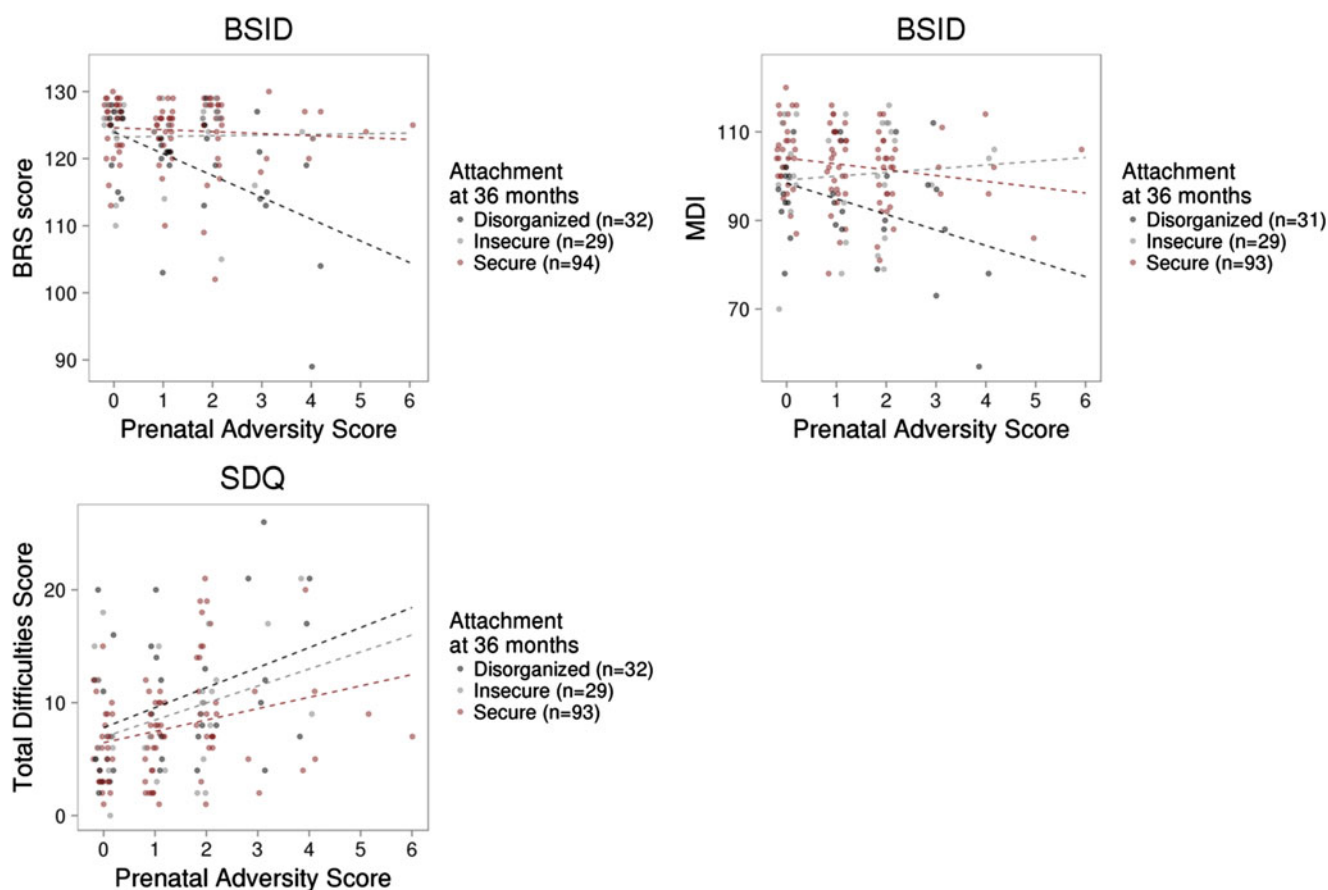
Figure 5. Prenatal adversity, infant attachment, and child outcome. (a) Prenatal adversity interacted with infant attachment to predict the Behavior Rating Scale score of the Bayley Scale of Infant Development (BSID) at 36 months. (b) The interaction between prenatal adversity and infant attachment did not significantly predict the Mental Development Index of the BSID, or (c) total difficulties from the Strengths and Difficulties Questionnaires in middle childhood (mean age = 6.99, SD = 1.33 years).

between the early life experience and the timing of female sexual maturation. The quality of parent–child relations in humans (Ellis & Essex, 2007; Ellis, Shirtcliff, Boyce, Deardorff, & Essex, 2011), including attachment insecurity (Belsky & de Haan, 2011), associate with the timing of female sexual maturation, consistent with life-history theories of early programming of human reproductive strategy under conditions of adversity (Chisholm, 1996). While the precise mechanism linking early family life to the timing of sexual maturation is unknown, there is evidence from Gene × Environment studies showing that the influence of early family life on the timing of menarche is moderated by *ESR1* variants (Hartman, Widaman, & Belsky, 2015; Manuck, Craig, Flory, Halder, & Ferrell, 2011). It will be of interest to determine if attachment-associated differences in *ESR1* DNA methylation covary with pubertal timing in females. Likewise, it will be important to determine if an altered rate of female sexual maturation contributes to the marked sex differences we observe in the association between infant attachment and variation in DNA methylation across groups.

Our findings highlight the genome as an important source of variation in DNA methylation of attachment-associated

VMPs, consistent with a large literature on genetic effects on DNA methylation (Hannon et al., 2016; Jaffe et al., 2016; Ng et al., 2017). Models that considered child genetic variation provided the best fit (adjusted R^2 or AIC) for approximately half of the attachment-associated VMPs. These findings are consistent with those of Teh et al. (2014), who report that the majority of variably methylated regions identified in umbilical cord samples from Singaporean neonates were best explained by gene–environment interactions. Such findings further highlight the need to consider genetic variation when seeking to characterize the biological embedding of early life experience.

In line with previous studies, we found that infant attachment predicted infant cognitive development (Bergman et al., 2010) but also predicted child behavior during this mildly stressful novel stimulus (i.e., the BSID). The association between infant attachment and both MDI and BRS scores represented medium to large effects. Consistent with Bergman et al., we also observed an interaction between a measure of prenatal adversity and infant attachment style to predict BSID scores. However, we note that unlike Bergman et al., our results were restricted to the BRS. It is possible that dif-

ferences in the age of assessment (17 months vs. 36 months) and our use of a three-group attachment classification system contributed to differences across our studies. However, we do note that the interaction between prenatal adversity and infant attachment is directionally consistent with the previous report (Bergman et al., 2010). As such, our data provide further evidence that attachment security may buffer the effects of prenatal adversity on measures of child outcome (Bergman et al., 2010), which may be of relevance for later psychopathology (McGoron et al., 2012).

We did not observe a significant association between infant attachment and child total mental health symptoms. Our data highlight the importance of adjusting for maternal mood when considering measures of child outcome based on maternal report. Likewise, we did not observe an interaction between prenatal adversity and infant attachment to predict child SDQ. It is possible that our choice of measure (SDQ) contributed to this null finding. In their meta-analysis Fearon et al. (2010) found a stronger association between infant attachment and child externalizing symptoms when assessed through observational methods rather than maternal report.

There are important limitations to this study. First, although this is the largest pediatric study of infant attachment and DNA methylation carried out to date, none of the individual VMPs associated with attachment were significant following adjustment for genome-wide correction. This is not surprising in light of the relatively small effect sizes observed between psychosocial adversity and variation in DNA methylation. Few studies in the field of biological psychiatry report changes in DNA methylation greater than 10%, despite functional effects of such changes on gene expression (Mehta et al., 2013). Our analysis was intended to determine whether infant attachment at 36 months of age was a significant source of interindividual variation in genome-wide DNA methylation. We also attempted to limit false-positive findings by including only those probes that showed the interindividual differences (i.e., VMPs). Likewise, due to our use of a three-group classification for infant attachment, our secondary analyses, which considered interactions with prenatal adversity and biological sex, are likely underpowered. As such, these data should be considered preliminary until replicated in an independent sample.

Second, the correspondence between buccal and neuronal methylation profiles is largely unknown. However, it is possible that regions of the genome that are sensitive to steroid hormones, which readily pass the blood–brain barrier, may produce coordinated modifications of DNA methylation across multiple tissues. The increasing availability of publicly available data sets and online tools (Edgar, Jones, Meaney, Turecki, & Kobor, 2017), which examine the concordance of DNA methylation across peripheral and central tissues, will facilitate the interpretation of findings from studies that use surrogate tissues for brain-based phenotypes.

In summary, our data show that the early care environment covaries with variation in genome-wide DNA methylation in middle childhood. The study of the DNA methylome provides a direct measure of the potential impact of the social environment at the level of the individual child and suggests a persisting influence of infant attachment on the DNA methylome. A deeper understanding of the molecular architecture of infant attachment will help monitor the efficacy of attachment-based therapeutic interventions, which seek to mitigate the lasting effects of early adversity on child development.

Supplementary Material

To view the supplementary material for this article, please visit https://doi.org/10.1017/S0954579418000627.

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