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Differential DNA methylation in peripheral blood mononuclear cells in adolescents exposed to significant early but not later childhood adversity

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Abstract

Internationally adopted adolescents who are adopted as young children from conditions of poverty and deprivation have poorer physical and mental health outcomes than do adolescents conceived, born, and raised in the United States by families similar to those who adopt internationally. Using a sample of Russian and Eastern European adoptees to control for Caucasian race and US birth, and nonadopted offspring of well-educated and well-resourced parents to control for postadoption conditions, we hypothesized that the important differences in environments, conception to adoption, might be reflected in epigenetic patterns between groups, specifically in DNA methylation. Thus, we conducted an epigenome-wide association study to compare DNA methylation profiles at approximately 416,000 individual CpG loci from peripheral blood mononuclear cells of 50 adopted youth and 33 nonadopted youth. Adopted youth averaged 22 months at adoption, and both groups averaged 15 years at testing; thus, roughly 80% of their lives were lived in similar circumstances. Although concurrent physical health did not differ, cell-type composition predicted using the DNA methylation data revealed a striking difference in the white blood cell-type composition of the adopted and nonadopted youth. After correcting for cell type and removing invariant probes, 30 CpG sites in 19 genes were more methylated in the adopted group. We also used an exploratory functional analysis that revealed that 223 gene ontology terms, clustered in neural and developmental categories, were significantly enriched between groups.

Studies in a variety of species have shown that adverse experiences early in life can have long-term effects on development (Meaney & Szyf, 2005). These early effects are broad ranging, including effects on brain structure and function (Nishi, Horii-Hayashi, & Sasagawa, 2014), immune deficiencies and elevated inflammatory factors (Lewis, Gluck, Petitto, Hensley, & Ozer, 2000; Lubach, Coe, & Ershler, 1995), heightened defensive responding (Meaney & Szyf, 2005), and impaired parenting behavior (Fleming et al., 2002). In human development, adverse childhood experiences are associated with a broad range of poor health behaviors and outcomes (Felliti et al., 1998; Shonkoff, Boyce, & McEwen, 2009). These negative outcomes include increased risk of elevated inflammatory factors and inflammation-related disorders (Coelho, Viola, Walss-Bass, Brietzke, & Grassi-Oliveira, 2014), alcohol abuse (Brady & Back, 2012), mental health disorders (Moffitt & Tank, 2013), and poor educational outcomes (Romano, Babchishin, Marquis, & Fréchette, 2014). Some children exhibit resilience (Cicchetti, 2013), and while we are still searching for the pathways between different types of early adversity and specific outcomes (Humphreys & Zeanah, 2015), the ubiquity of the effects and the long developmental reach has led to an interest in understanding how these experiences "get under the skin" to affect behavioral and physiological development (Hertzman & Boyce, 2010).

Children placed in institutional (orphanage) care early in life and then adopted or fostered into families have served as an important group informing our understanding of neural and physiological correlates of early adverse experiences. These children have experienced a variety of adverse early life conditions, often beginning at conception, that may include impoverishment, exposure to pathogens, abuse, and

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neglect (Gunnar, Bruce, & Grotevant, 2000). Their experiences overlap with those of noninstitutionalized children reared in poverty. However, unlike many children reared in adverse conditions, the early and later life experiences of these children differs markedly, allowing a greater understanding of what might be instantiated in biology early that is not changed by placement in well-resourced and supportive families (Zeanah, Gunnar, McCall, Kreppner, & Fox, 2011). On average, families of internationally adopted children in this study are in the upper 20% of family income, and typically both parents have at least a college education (Hellerstedt et al., 2008). Observations of the parenting in families who adopt internationally have revealed high levels of sensitivity and responsiveness (Garvin, Tarullo, Van Ryzin & Gunnar, 2012). However, parents of children with significant developmental delays are more controlling and directive (Garvin et al., 2012), until the children begin to catch up developmentally (Croft, O'Connor, Keavene, Groothues, & Rutter, 2001). In previous work on the formation of attachment among children adopted from orphanages, our group found that within 8 months of adoption nearly all (95%) have formed an attachment to their adoptive parents, 70% of these are secure, although about 23% are disorganized/disordered patterns of attachment behavior (Carlson, Hostinar, Mliner, & Gunnar, 2014). Consistent with a generally supportive and well-resourced environment, after adoption children exhibit remarkable physical and cognitive catch-up growth; however, long-term deficits in a variety of domains still remain, including theory of mind, executive functions and attention regulation, and emotion regulation (Kumsta et al., 2010; Loman, Wiik, Frenn, Pollak, & Gunnar, 2009; Zeanah et al., 2003).

Numerous processes may account for the capacity of early exposures to produce long-term effects even when the period of early adversity lasts only a few years and the time spent in low-risk environments consumes most of the child's developing years. These processes may include learned patterns of behavior that influence children's perceptions of their experiences and responses they elicit from others (Stovall & Dozier, 2000), effects on brain development that influence learning capacity (Loman et al., 2013), and impacts on the developing defensive system that may limit children's engagement of their environment (Tottenham et al., 2010). To explain these findings, studies have been directed toward examinations of stress hormones (Koss, Hostinar, Donzella, & Gunnar, 2014), brain function (event-related potential; Loman et al., 2013; Vanderwert, Marshall, Nelson, Zeanah, & Fox, 2010), brain structure (Chugani et al., 2001; Hodel et al., 2015; Mehta et al., 2009; Sheridan, Fox, Zeanah, McLaughlin, & Nelson, 2012), and more recently, molecular processes (Drury et al., 2012). Of particular current interest is the possibility that early experiences influence later outcomes by sculpting the epigenome (Boyce & Kobor, 2015; Hertzman, 1999; Meaney & Szyf, 2005).

Epigenetics refers to modifications of the genome that affect DNA accessibility and potentially alter gene expression, but do not alter the base-pair sequence (Bird, 2007). One specific and well-understood epigenetic modification is DNA methylation, which consists of the addition of a methyl group to the cytosine in a C-G dinucleotide (CpG) of DNA. CpGs are nonuniformly distributed in the genome and tend to be clustered in regions referred to as CpG islands (Illingworth & Bird, 2009). Many genes have a promoter-associated CpG island, and DNA methylation of these islands is often correlated with gene expression levels (Jones, 2012; Weber et al., 2007). DNA methylation is also tightly linked to cell differentiation and identity, with cellular heterogeneity within a given tissue being one of the major predictors of epigenetic variability (Jaffe & Irizarry, 2014; Lam et al., 2012; Liu et al., 2013).

Recent research has suggested that DNA methylation acts as a principal mechanism by which early-life experiences affect neurobehavioral development (Boyce & Kobor, 2015). Early environments have been consistently associated with changes in DNA methylation across multiple mammalian species (Lutz & Turecki, 2014). In humans, studies of socioeconomic status suggest that experiencing low socioeconomic status throughout childhood is associated with altered DNA methylation (Borghol et al., 2012; Lam et al., 2012; McGuinness et al., 2012) and gene expression (Miller et al., 2009) later in life. Prospective, longitudinal research has shown that parental stress in infancy and early childhood associates with differential DNA methylation in adolescents (Essex et al., 2013). Research on the impact of childhood maltreatment on the epigenome has demonstrated changes in DNA methylation in the brain (McGowan et al., 2009) as well as peripheral tissues (for a review, see Lutz & Turecki, 2014). Most relevant to the work presented here, in one study with children in a Russian institution, genome-wide DNA methylation patterns in peripheral whole blood were examined (Naumova et al., 2011). Compared to children reared in poverty in their Russian birth families, those living in an institution showed increased DNA methylation across a number of CpG loci, particularly those located among genes related to immune regulation and cellular signaling.

The purpose of the present study was to examine internationally adopted youth who had been adopted out of conditions of adversity early in life into families in the upper Midwest of the United States. These youth were compared to similarly aged youth who had been born and raised in Midwest families of comparable wealth and education to those who adopt internationally. To keep ethnicity consistent, we studied only Caucasian youth. From conception until adoption, the youth's lives were markedly different; from adoption on they lived in comparable environments. Consistent with typical ages for international adoption from Russia/Eastern Europe, we anticipated that 2 years would be the average age at adoption. Thus, because we tested them in middle adolescence, roughly 80% of their lives would have been spent in comparable circumstances to the nonadopted youth. Our goal was to determine whether exposure to adverse conditions from conception through infancy left behind a signature of DNA methylation that remained up until middle adolescence.

Method

Participants

Participants included 50 adolescents adopted from institutions for orphaned or abandoned children in Eastern Europe or Russia (*M* age = 15.68 years, *SD* = 1.48, age range = 12.75–18.67 years; 25 females) and 33 adolescents of European descent who were raised in their biological families in the United States (*M* age = 15.41, *SD* = 1.24, age range = 13.01–17.25; 18 females). There were no significant differences in age, $t = 1.17$, $df = 82$, *ns*, or gender, χ^2 (1) = 0.80, *ns*. The differences in sample size were dictated by finances in this study, designed as a preliminary study of methylation patterns in children adopted from conditions of adversity. More adopted than nonadopted children were tested to allow the opportunity to examine early experience correlates within the adopted group.

Exclusion criteria in both groups included a diagnosis of autism, fetal alcohol syndrome, Down syndrome, or other major congenital disorder. Adolescents in the adopted group came into their adoptive families on average at 21.8 months (range = 6–78 months, *SD* = 17.16) and had spent 91.6% (range = 56%–100%, *SD* = 12.79) of their preadoption lives in institutional care. The reasons for placement in institutional care were not always known to the adoptive families; however, 71% of the adopted youth had never lived anywhere else, another 20% were abandoned by or removed from their families by 6 months of age, and 10% had spent more than 6 months in some kind of noninstitutional setting prior to entering these institutions. Countries of origin included Russia (34), Romania (6), Ukraine (4), Bulgaria (1), and other Eastern European countries (5).

Adopted youth were recruited from a registry of families of internationally adopted children who were interested in research. The registry reflects approximately 60% of all internationally adopted children in our catchment area. Youth in this study had previously been in an imaging study when they were 12 and 13 years of age (Hodel et al., 2015). In addition to the exclusion criteria noted above, the adopted youth had also met inclusion and exclusion criteria for magnetic resonance imaging research. The nonadopted, comparison youth were recruited by phone from a registry of families interested in participating in research that was initiated through letters mailed to parents of all live births in our catchment area. Written consent and assent were obtained from the adolescents, and their families received monetary compensation. All procedures were approved by the University of Minnesota Institutional Review Board.

The comparison group was selected to roughly match the adopted group on family income and parent education. At the time of testing, adolescents from both groups lived in families of similar socioeconomic levels, with both groups averaging pretax incomes between $85,001 and $100,000 per year and parental educational levels of a bachelor's degree or higher.

Six participants were not able to provide samples, or their samples were excluded because of problems with collection. No youth who had a fever on the day of sampling were included. Sample size and demographics above were reported with these individuals excluded.

Procedure

Adolescents and their primary caregiving parent attended a 2-hr laboratory testing session that included the completion of questionnaires and a blood draw between 9:00 a.m. and 11:00 a.m. Following arrival at the testing site, consent and assent were obtained in a private room, and participants received monetary compensation. Parents and adolescents completed questionnaires in individual rooms, and a single vial of blood (7 ml) was obtained from the adolescent by antecubital venipuncture. Within 1 hr of blood draw, samples were transported to the laboratory, and peripheral blood mononuclear cells (PBMCs) were isolated as previously described (Miller et al., 2009). PBMC pellets were frozen and stored at –80 °C until DNA extraction.

Measures

Demographic questionnaire. Parents completed questions about demographic information (e.g., income and education), lifetime history of the adolescents' medical and psychiatric diagnoses, current treatments and services, and preadoption history (where applicable).

McArthur Health and Behavior Questionnaire (HBQ). The HBQ (Essex et al., 2002) was completed by both parents and youth. The parent version consists of 124 items, and the child version consists of 164 items, that probe physical and mental health, as well as functioning in academic and social domains. Parent reports of psychiatric diagnoses and current psychotropic medications and parent and youth reports on health problems on the day of testing and in the last month from this measure were used in analyses.

The Life Events Checklist—Child/Adolescent Version. This checklist (Johnson & Cutcheon, 1980) was administered to parents and youth to assess stress during the past year. Adolescents reported whether any of 46 events happened to them, whether the event was bad or good, and what level of impact the event had on them. Negative events with no impact were coded as 0 (*some* = 1, *moderate* = 2, *great* = 3), and the total of these events was summed for a total number of negative life events in the past year. Parent and youth reports were correlated ($r = .42$, $p < .001$) and were averaged to stabilize the measure.

DNA methylation

DNA was extracted from PBMC pellets using the DNeasy kit (Qiagen, Germantown, MD, USA). Bisulfite conversion of DNA was performed with the EZ-DNA methylation kit (Zymo Research, Irvine, CA, USA). Bisulfite converted

DNA was interrogated with the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc, San Diego, CA) according to manufacturer's instructions. Background subtraction and color correction of data was performed using Illumina GenomeStudio software, at which point data were imported into R for further preprocessing (R Development Core Team, 2008).

Data preprocessing

Technical replicates and expected sex of each participant were checked to ensure consistency. Next, the 65 single nucleotide polymorphism probes, 11,648 X/Y chromosome probes, 2,751 probes not detected above background in at least one sample, 15,747 probes with fewer than three beads contributing to the signal in at least one sample, and 39,440 probes we have previously shown to exhibit poor design features were filtered out, leaving a final total of 415,926 probes (Price et al., 2013). DNA methylation data was quantile normalized using the lumi package, and SWAN normalization was performed to correct for probe type (Du, Kibbe, & Lin, 2008; Maksimovic, Gordon, & Oshlack, 2012). Finally, ComBat was performed on the data to remove chip and row effects sequentially (Chen et al., 2011). Both iterations of ComBat included adoption status and sex as additive effects in the model, identifying them as important variables for which variance should be protected.

Cell type composition of samples was determined using published methods (Houseman et al., 2012; Koestler et al., 2013). Data was adjusted for cell composition by fitting probewise linear models using predicted cell composition as variables and scaling the DNA methylation data using the resulting residuals (Jones, Islam, Edgar & Kobor, 2015). Principal component analysis (PCA) was performed on a matrix of normalized M values using the prcomp function with centering in R. Resulting loadings were correlated with sample variables using a Spearman correlation.

Data analysis

We examined the data for potential covariates by determining which variables might be significantly different by group. Those differing by group were then subjected to further analyses to determine whether they also were associated with DNA methylation. These latter analyses were performed using the limma package (Smyth, 2005). After determining the covariates needed in the analyses, a differential DNA methylation analysis was performed on all probes on the array, again using the limma package (Smyth, 2005). This was done on the noncell-type-adjusted data to determine the effect of not correcting for these important differences (Smyth, 2005). Permutations were performed in exactly the same way, except that group assignments were randomized 100 times, and resulting *p*-value distributions plotted.

To conserve power, because it has been shown that most sites of DNA methylation are invariable across individuals (e.g., Smith et al., 2015), we performed a filtration step to remove any invariable sites, defined as having a standard deviation of less than 0.05, or 5% methylation. This step removed 407,156 sites, resulting in a final list of 8,770 variable sites. These invariable sites have been repeatedly identified in the literature as making up the majority of total CpGs, and generally represent constitutively methylated or unmethylated sites that are less likely to be associated with gene expression, and this filtration has been used in a number of studies (Bourgon, Gentlemen, & Huber, 2010; Smith et al., 2015; Teh et al., 2014; Wagner et al., 2014). Thus, this step ensures that multiple test correction will not be unduly penalized for measurements with no underlying variability. Analysis on this filtered data set was performed using the lm function from the stats package with age, sex, and negative life events as covariates.

Functional enrichment analysis

We matched each probe to a single gene name in the following manner: (a) sites with no Illumina-annotated UCSC_refgene name were annotated as NA; (b) sites with one or more gene name entries in the Illumina-annotated UCSC_refgene_name and where all gene names were identical were annotated to the given gene; and (c) sites with multiple gene name entries in the Illumina-annotated UCSC_refgene_name and where gene names differed were annotated to the closest transcription start site based on the annotation in the Closest_TSS_gene_name column from a published reannotation (Price et al., 2013). A gene ontology (GO) analysis was performed on uncorrected *p* values using ErmineJ, and gene names were ranked by lowest *p* value for any associated CpG (Gillis, Mistry, & Pavlidis, 2010). ErmineJ parameters were as follows: gene score resampling method, 5–100 cluster size, best scoring replicates, negative log of *p* values, and mean for clusters. For functional clustering, ErmineJ output was separated into a gmt file containing GO terms and associated genes, and a text file containing ranked GO terms, false discovery rate (FDR) adjusted *p* values, and multifunctionality values, which were added to the normal FDR *p*-value column for graphical purposes. These files were input into cytoscape for clustering analysis using EnrichmentMap with the following parameters: $p = .05$, FDR $p = 0.99$ (here representing multifunctionality), Jaccard and Overlap combined coefficient 0.5. Only clusters with five or more members were visualized. Each resulting cluster was named using WordCloud, where the top ranking word was used, unless a second word was required to make an understandable term in which case both were used.

Results

Determining covariates

In order to create an appropriate model by which to test DNA methylation differences associated with adoption status, we examined potential covariates. Numerous variables were exam-

ined for group differences. Those exhibiting differences were parent (but not youth) report of internalizing, externalizing, and attention-deficit/hyperactivity disorder (ADHD) symptoms, Hotelling F (3, 78) = 5.18, $p < .01$. Groups differed in psychiatric diagnoses with 41% of the adopted and only 13% of the nonadopted group carrying any psychiatric diagnosis, χ^2 (1, $N = 83$) = 7.44, $p < .01$. Most often for the adopted youth the diagnosis was ADHD, with 31% of the adopted and 3% of the nonadopted carrying that diagnosis, χ^2 (1, $N = 83$) = 9.29, $p < .01$. There were no group differences in diagnoses of depression or anxiety ($ps > .10$). Consistent with differences in diagnoses, adopted adolescents (31%) were more likely to be taking psychotropic medication than were nonadopted youth (13%), χ^2 (1, $N = 83$) = 3.77, $p = .05$. We averaged parent and youth report for negative life events with the impact scores for the time period the children were in their current families. There were no significant group differences, F (1, 81) = 0.28, *ns*. This was also true for negative events in the past 12 months as an average of parent and child report, t (81) = –0.19, $p = .85$. Finally, health problems reported in the last month yielded a nonsignificant trend toward significance, with 11% of nonadopted and 25% of adopted youth experiencing one or more health problems in the month prior to testing, χ^2 (1, $N = 83$) = 3.2, $p = .07$.

We then examined these variables to determine their association with DNA methylation. We found no evidence that any of the parent-reported scores for internalizing, externalizing, or ADHD, or whether the child was diagnosed or being medicated for a psychiatric disorder was associated with DNA methylation. Only negative life experiences was shown to have a signature independent of other covariates, so it, along with age and sex, was included as a covariate in all subsequent analyses. Negative life experiences showed two CpGs associated at an FDR of 0.05, but neither showed a mean difference between groups of greater than 2%.

Cell-type analysis

Because DNA methylation is highly influenced by cell type, it is essential to account for interindividual differences in white blood cell distributions between groups. Thus, we next examined whether adoption status was associated with cell-type distribution. At the time of blood draw, no complete blood count data were collected, so we used a published algorithm to backpredict the underlying cell types from the DNA methylation data (Houseman et al., 2012). Because recent illness can affect the immune cells in circulation, we tested whether the 17% of participants who had experienced health problems in the last month differed in cell-type composition from those who had not. None of the t tests were significantly different ($ps > .20$). We next tested whether cell-type composition differed between adopted and nonadopted participants, and found a marked difference between the groups (Figure 1a). We compared each cell type across the two groups using an unpaired, two-tailed t test. As shown, there were significantly fewer CD4+ T cells and more CD8+ T cells in the adopted compared to the nonadopted youth. In addition, B cells were lower in frequency in the adopted than in the nonadopted youth. These analyses indicate how essential it is to correct for interindividual differences in blood cell-type composition in order to avoid spurious DNA methylation findings; hence, in subsequent analyses cell type was controlled.

To further explore factors that might be driving this cell-type difference, we focused on the CD4/CD8 ratio, which may reflect immune competence. We examined it in relation to each of the potential covariates that differed by group. No significant correlations were obtained for use of psychiatric medication, average negative life events, and negative life events in the last 12 months ($dfs = 79$, $rs < .15$, $ps > .20$). Youth with more externalizing symptoms, r (79) = –.26, $p < .05$, and ADHD symptoms, r (79) = –.35, $p < .01$, did exhibit a lower CD4/CD8 ratio. However, when we entered these factors as covariates, the group difference between adopted and nonadopted youth was still highly significant, F (1, 76) = 11.33, $p < .001$. It should be noted that while none of the youth had a fever on the day of testing, some reported mild cold or allergy symptoms. These did not differ by group, but were correlated with CD4/CD8 ratios, r (79) = .35, $p < .001$.

The finding of cell-type differences was remarkable in itself. Thus, before attributing these differences to institutional care, we took advantage of a previously published study that also examined effects on DNA methylation of institutionalization in Eastern European children that used the older 27k array (Naumova et al., 2011). That study compared children in institutions to similarly impoverished children living in their families in Russia. The researchers supplied us with their data, and we applied the same backprediction algorithm (Houseman et al., 2012). We did not observe the same CD4/CD8 T cell ratio between groups, and noted that both groups of impoverished Russian children had lower CD4/CD8 ratios than the nonadopted US-born group and comparable to the adopted Russian/Eastern European group (Figures 1b and 1c).

DNA methylation analysis without cell-type correction

Given that we knew that differences in cell-type composition existed between our groups, we sought to compare DNA methylation differences between the groups with and without cell-type correction, to emphasize the importance of this step. This analysis is timely, considering that correcting for cell type has only recently emerged as an important aspect of epigenetic population studies. We used PCA on the noncell type-corrected data. This PCA (Figure 2a) revealed large DNA methylation signals associated not only with adoption group, sex, age, and negative life experiences but also with cell types. We then proceeded to determine specific DNA methylation changes associated with adoption status, controlling for age, sex, and negative life events. Without correcting for cell type, using a Storey (2003) q value of 0.1 and minimum group mean beta value difference of 0.02, 136,339 sites

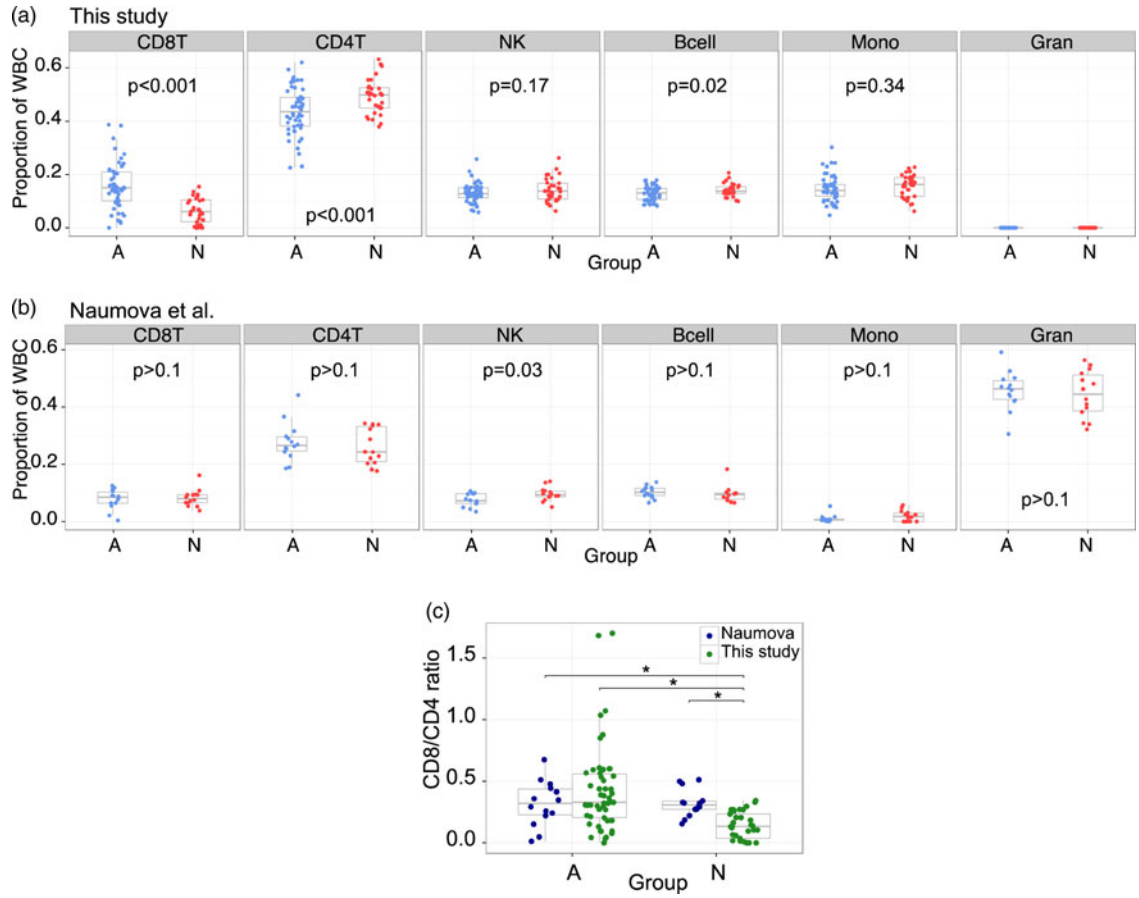

Figure 1. (Color online) White blood cell type proportions differed between adopted and nonadopted youth in this study but not in a previously published data set. Proportion of white blood cell types as predicted using a published algorithm are shown separated by adopted (blue online only) and nonadopted (red online only) in (a) our study and (b) a previously published study (Naumova et al., 2011). Boxes are box and whisker plots of the 25th, 50th, and 75th percentile. Indicated *p* values are the result of unpaired *t* tests comparing the distribution of the two groups. CD8T, CD8+ T cells; CD4T, CD4+ T cells; NK, natural killer cells; MONO, monocytes; GRAN, granulocytes. Note that no granulocytes were estimated because these are mononuclear cells. (c) Ratios of CD8/CD4 T cells differed between the nonadopted participants in this study and the other groups.

were differentially methylated between the two groups (data not shown). We tested these sites to compare overlap with sites that were previously observed to be differentially methylated by cell type, and of the 136,339 sites, 109,961 were found in a previous paper to be associated with one or more cell type at a false discovery rate of 0.05 (Jaffee & Irizarry, 2014). This implies that, for studies where cell-type proportions are correlated with the variable of interest, the majority of hits are sites that differ directly because of cell-type composition and not the variable if cell composition is not taken into account.

DNA methylation analysis with cell-type correction

We then repeated these analyses on data that had been corrected for cell-type composition. In the PCA, after regressing out differences due to cell-type composition, the signal associated with adoption status was greatly reduced in both significance and magnitude of variance (Figure 2b). DNA methylation signals associated with age, sex, and negative life events remained, but at a much lower proportion, as would be expected given age, sex, and stress associations with T cell composition (Pérez-de-Heredia et al., 2015; Stefanski & Engler, 1998; Figure 2b). We next performed our linear modeling on this cell type-corrected data, using the same covariates, age, sex, and negative life experiences. With the same significance criteria (i.e., a *q* value of 0.1 and minimum group mean beta value difference of 0.02), no sites were significantly different between adoption groups. However, the unadjusted *p* values showed a pronounced leftward skew (Figure 2c, red line online only). We permuted the group assignments 100 times and repeated the linear modeling, which resulted in a predominantly flat background distribution of *p* values, indicating that our left skewed distribution showed enrichment for low *p* values beyond the expectation by chance (Figure 2c, black line).

Because it has been shown that most sites of DNA methylation are invariable across individuals (e.g., Smith et al., 2015), we next performed a filtration step to remove any invariable sites. This filtration step removed any site with a

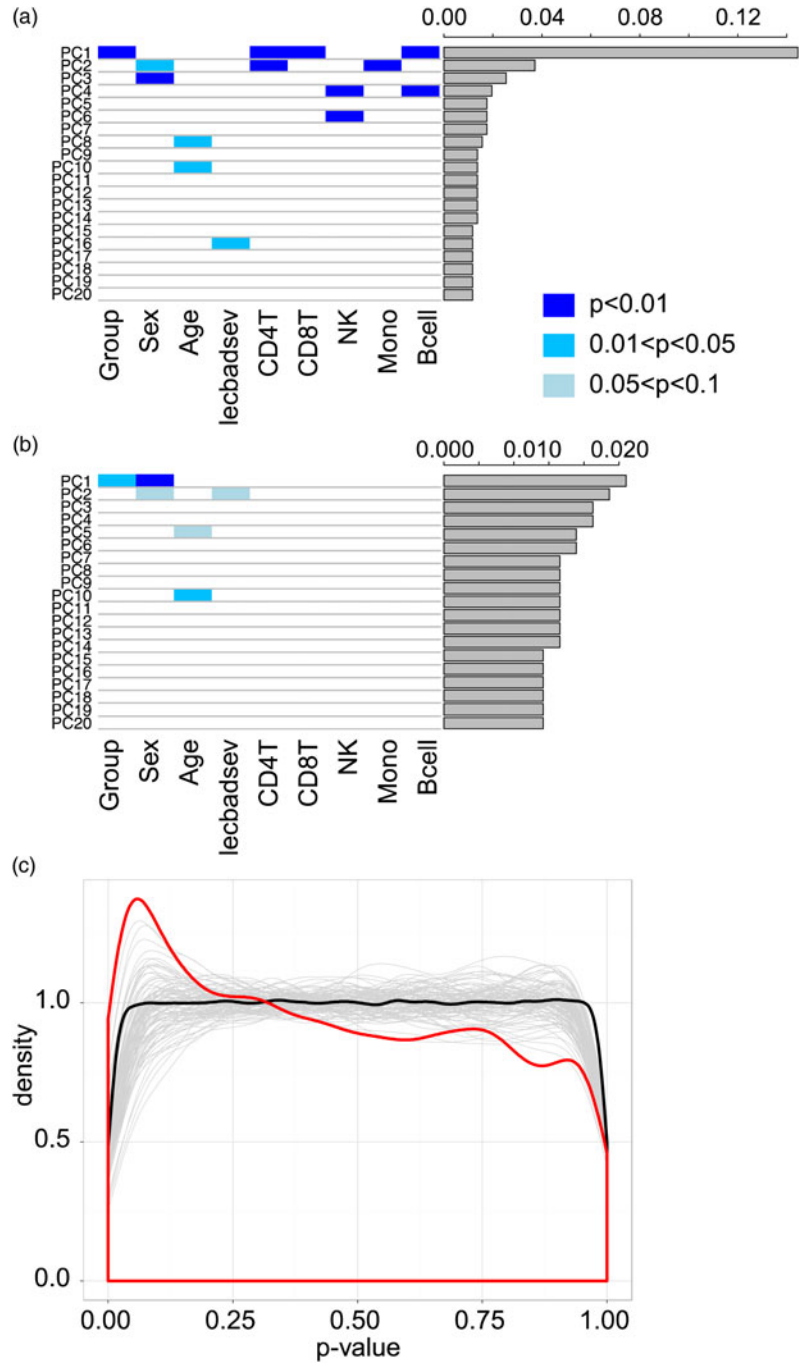

Figure 2. (Color online) Blood cell type is an important and confounding factor in the analysis of DNA methylation and adoption status. On the left, heat maps of significance between top 20 principal component scores and variables of interest. On the right, histograms of the proportion of variance accounted for in the data by the indicated principal component. (a) Prior to correction for cell type, principal components were significantly associated with adoption status (group), the covariates of interest, and all cell types. (b) After correction for cell type, association with cell types is no longer present, and both significance level and proportion of variance for the group-associated principal component has decreased. (c) Density plot of uncorrected *p*-value distribution for linear model on 415,926 probes (red line online only). Left skewing indicates enrichment for very small but nonsignificant *p* values. Gray lines represent similar distributions for 100 permutations of group assignment, summarized by a mean line in black.

standard deviation of less than 0.05, and resulted in a final list of 8,770 variable probes. This filtering removed sites with no underlying variability, thus reducing the burden of multiple test correction and increasing the power to identify the sites responsible for the leftward skew in Figure 2c. Using *q* value of 0.1 and minimum mean difference of 2% methylation between groups, there were 30 differentially methylated sites in 19 different genes. Genes with more than 1 CpG found to be differentially

methylated included *TMEM200C* (4), *PPP1R3G* (3), *GLYATL2* (2), *CYP1A1* (2), and *miR-324* (2). Table 1 contains a full list, including specific gene functions, definitions, and mean difference between groups. None of these 30 CpGs showed correlation between DNA methylation beta value and percent composition of any cell type in our samples (maximum absolute Spearman $\rho = 0.17$).

We next set out to compare our results to the previously published data set used for cell-type analysis above (Naumova et al., 2011), We identified an overlap of four CpGs (cg00520135/*TPM1*, cg05968233/*ALKBH7*, cg06938878/*CALCB* and cg20359349/*FAM215A*) between our 30 hits and the CpGs present on the 27k array used in the previous study. None of these four showed differential DNA methylation between adopted and nonadopted participants in that data set. We also examined the 914 sites the previous study identified as significantly differentially methylated between groups (Naumova et al., 2011). Of these, 213 were in our list of 136,339 significantly different sites before the cell-type correction. Only 64 of the remaining showed variability in our sample, and none were differentially methylated between groups in our data set. There are many possible reasons for this lack of replication, including different types or severity of early life experiences and the fact that the Naumova participants were still living under conditions of adversity and our participants had not been living under those conditions for many years.

DNA methylation at one of the genes in our hit list, *CYP1A1*, has previously been associated with exposure to cigarette smoke. Children in orphanages in Russia and Eastern Europe are frequently exposed to cigarette smoke in the institutions (Gunnar et al., 2000). To determine whether this would be a reasonable interpretation of this finding, we further examined the *AHRR* gene, which is the gene with the best replicated finding of DNA methylation due to smoke exposure. We examined 11 papers that had published associations between methylation and *AHRR* and cigarette smoke, and determined that five CpGs were found in more than 3 of these studies (Dogan et al., 2014; Elliott et al., 2014; Joubert et al., 2012; Lee et al., 2015; Markunas et al., 2014; Monick et al., 2012; Novakovic et al., 2014; Richmond et al., 2015; Shenker et al., 2013; Sun et al., 2013; Tsaprouni et al., 2014). Four of these five CpGs were present in this data set, so we tested their DNA methylation using the same linear model as the whole genome analysis. Two of these four sites in *AHRR* (cg05575921 and cg23067299, $ps < .01$) showed differential DNA methylation based on adoption status, in the expected direction of change based on higher cigarette smoke exposure in adopted than nonadopted children (see Figure 3). This implies that the observed differential methylation in *CYP1A1* may be due to our adopted participants having had higher early life exposures to cigarette smoke. Of course, it could also be that the adopted youth were more likely to be smoking currently than were the nonadopted youth. Although we did not have a measure of youth smoking, we did have the parent report of externalizing problems that did differ by group and is, by definition, associated with conduct problems in adolescence such as illegally smoking. When we added externalizing symptoms as a covariate in the analysis of *CYP1A1*, it did not reduce the association with group.

Functional analysis

As a final exploratory analysis, we performed a functional analysis as described in the Methods. The specific method used, ErmineJ, requires the full, unfiltered data set to uncover broader epigenetic signatures related to these groups. We mapped each probe on the 450K array to a unique gene name and created a background list of all genes present in the 415,926 remaining probes and their associated GO terms. We then tested for significant enrichment of particular GO terms at the top of a list of genes ranked by their uncorrected *p* value for differences between groups. Two hundred and twenty-three GO terms were significantly enriched at the stringent program-selected false discovery rate of 0.05. Important to note in this analysis is the phenomenon of multifunctionality. Multifunctional genes tend to be highly studied and thus associated with many GO terms, so the presence of multifunctional genes at the top of a list would tend to lead to a large number of GO terms showing significance.

To further refine this functional analysis and group these GO terms in larger functional units, we clustered the GO terms according to shared genes (Merico, Isserlin, Stueker, Emili, & Bader, 2010; Shannon et al., 2003). The resulting functions can generally be clustered into two categories: neural (neuron, behavioral response, action potential, and the large regulation cluster) and developmental (organ induction, nephron tubule development, pattern specification, bone morphogenic proteins signaling, and the large morphogenesis cluster; Figure 4). Given the large number of multifunctional genes in this list, it was not surprising that many of the GO terms were also highly multifunctional.

Discussion

The goal of this study was to examine differences in the epigenomes of immune cells in peripheral blood in adolescents adopted as young children from conditions of significant adversity. This adversity took the form of early adversity these youth had experienced from birth to women in Russia/Eastern Europe who gave up their parental rights and placed their infants in institutions/orphanage where they were reared for the first few years of life. Following this, the children were adopted into well-resourced families in the United States. Initially, we estimated the underlying white blood cell composition in these participants with the goal of controlling for it, and noted drastic differences in CD4T/CD8T cell ratios. After correcting for these cell-type differences and restricting the analysis to variable CpG loci, 30 sites on 19 genes met criteria for differential DNA methylation by adoption group. We also conducted a functional analysis of GO terms and found that group differences clustered in two areas: neuronal and

Table 1. *Thirty differentially methylated genes between adopted and nonadopted youth ordered by number of sites and chromosome*

Gene Symbol	No. of CpGs	Delta Beta	Chr.	Name	Function
TMEM200C	4	−0.046[a]	18	Transmembrane protein 200C	Transmembrane protein with no specifically identified function
PPP1R3G	3	−0.042[a]	6	Protein phosphatase 1, regulatory subunit 3G	Involved in glucose homeostasis and glycogenesis in the liver (Luo et al., 2011; Zhang et al., 2014)
GLYATL2	2	−0.044[a]	11	Glycine-*N*-acyltransferase-like 2	Acetylation-regulated glycine acyltransferace expressed in salivary gland, trachea, spinal cord, and skin (Waluk et al., 2010; Waluk, Sucharski, Sipos, Silberring, & Hunt, 2012)
CYP1A1	2	−0.042[a]	15	Cytochrome P450, family 1, subfamily 1, polypeptide 1	Cytochrome linked to drug metabolism, genetic variants associated with lung cancer risk, and methylation associated with exposure to cigarette smoke (Joubert et al., 2012; Shenker et al., 2013)
miR-324	2	−0.048[a]	17	MicroRNA 324	MicroRNA with two validated targets, GLI1 and SMO, both involved in neuron differentiation (Ferretti et al., 2008)
SFRP2	1	−0.037	4	Secreted frizzled-related protein 2	Wnt family receptor regulating apoptosis response to tumor necrosis factor; evidence of epigenetic silencing in cancer (Melkonyan et al., 1997; Perry et al., 2013; Saito et al., 2014)
ADAMTS2	1	−0.056	5	ADAM metallopeptidase with thromobospondin type 1 motif, 2	Highly processed enzyme with multiple isoforms and different functions in collagen metabolism; mutations in this gene have been associated with Ehlers–Danlos syndrome type VIIC, a connective tissue disorder (Colige et al., 2004, 2005)
ESM1	1	−0.047	5	Human endothelial cell-specific molecule-1	Endothelial cell-specific and cytokine-regulated secreted protein involved in immunity and inflammation (Lassalle et al., 1996; Lee, Ku, Kim, & Bae, 2014)
Mir-873	1	−0.048	5	MicroRNA-873	Regulator of inflammatory cytokines in white blood cells and brain
CFTR	1	−0.057	7	Cystic fibrosis transmembrane conductance regulator	Ion channel expressed in multiple tissues, responsible for cystic fibrosis (Riordan et al., 1989)
CALCB	1	−0.032	11	Calcitonin-related polypeptide beta	Putative secondary peptide expressed from the CALC locus, which produces both calcitonin and a calcitonin gene-related neuropeptide, both expressed in brain and involved in inflammation (Hou et al., 2011)
OR4C13	1	−0.052	11	Olfactory receptor, 4 C 13	Olfactory receptor (Malnic, Godfrey, & Buck, 2004)
ATP8A2	1	−0.045	13	Aminophospholipid transporter, class I, type 8A, member 2	A protein-coding gene involved in phospholipid-translocating ATPase activity expressed in the brain and retina (Coleman et al., 2014)
AJ278121	1	−0.039		NA	Unannotated ORF
TPM1	1	−0.049	15	Tropomyosin alpha chain 1	An actin-binding protein involved in the contractile system of striated and smooth muscles and required for proper cardiac development (McKeown, Nowak, Gokhin, & Fowler, 2014)
FAM215A	1	−0.043	17	Family with sequence similarity 215 member A	Unannotated possible ncRNA
LOC730755	1	−0.042	17	Unnamed locus	Uannotated locus with sequence similarity to keratin associated protein
ALKBH7	1	−0.035	19	AlkB, *E. Coli*, homolog of, 7	Involved in alkylation and oxidation induced necrosis and obesity (Wang et al., 2014)
CERK	1	−0.091	23	Ceramide kinase	Kinase involved in the metabolism of sphingolipids, shown to be involved in immunity and inflammation (Bornancin, 2011)

[a]Indicates average delta beta of all underlying CpGs.

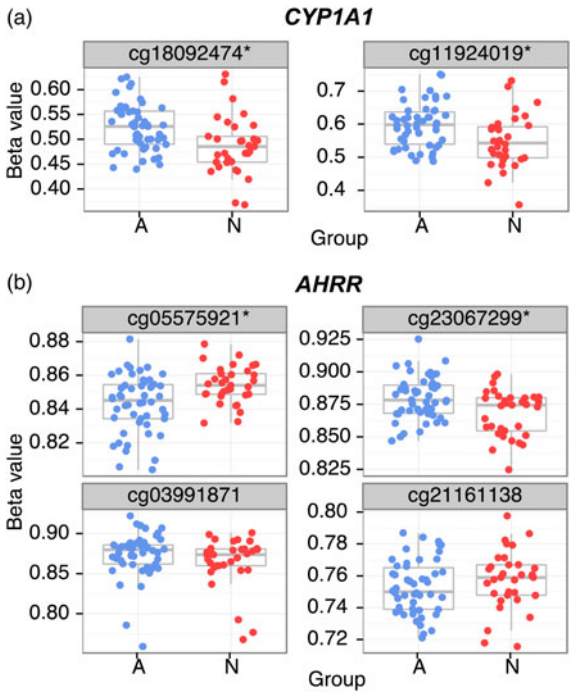

Figure 3. (Color online) DNA methylation findings were supportive of higher cigarette smoke exposure in adopted participants. (a) *CYP1A1* was more methylated in adopted (A, blue online only) than nonadopted (N, red online only) participants at two sites as found in the epigenome-wide association study analysis. (b) To validate this, four CpGs in the *AHRR* gene were examined for differential DNA methylation between adopted and nonadopted participants. Two, cg05575912 and cg23067299 (indicated with an asterisk), were significantly differentially methylated at a false discovery rate of 0.05.

developmental. These results are consistent with behavioral and health differences frequently noted for children adopted from this region of the world following orphanage rearing (Kumsta et al., 2010; Loman et al., 2009; Zeanah et al., 2003). However, there are a number of reasons to view these data as only suggestive of early adversity effects as the many differences between the adopted and nonadopted youth leave open other explanations that will be discussed below.

Before discussing the DNA methylation findings, it is important to note both the cell-type differences and the effects of correcting versus not correcting for those differences. The adopted and nonadopted youth differed strikingly in the type of immune cells in circulation. Specifically, the adopted youth exhibited fewer CD4+ and more CD8+ cells than the nonadopted youth and fewer B cells. This pattern suggests a reduced immune competence that may have some adaptive significance in the environment in which these children were conceived and reared prior to adoption. The difference was not explained by significant health differences in the month prior to blood sampling, nor was it explained by externalizing and ADHD symptoms, even though these variables differed between groups and correlated with the CD4/CD8 ratio. In the field of psychoimmunology, many animal studies have documented that a variety of early adversity affects the differentiation of T cells within the thymus and their circulating numbers (Eriksen et al., 2014). Reduced CD4+ and increased CD8+ T cells in circulation also has been noted recently in adolescent Rhesus monkeys who were maltreated as infants (Kohn et al., 2014). As in the present study, living conditions were comparable for the maltreated and comparison monkeys after weaning. The present finding is also consistent with evidence that postinstitutionalized youth are impaired in containing the Herpes simplex virus with titers that are even higher than those of youth in child protection for physical abuse (Shirtcliff, Coe, & Pollak, 2009).

However, as noted earlier, in the present study many factors from conception until adoption differed between the adopted and nonadopted children. Only once the children were adopted were the two groups comparable in their physical and social care. To be sure that we did not misattribute the cell-type differences, or other methylation differences, to institutional care versus the general context of being born into poverty in Russia/Eastern Europe, we obtained the DNA methylation information from a study of impoverished Russian children, some raised by their families and some living in Russian orphanages (Naumova et al., 2011). Using the same techniques to backpredict cell type that we used on our data (Houseman et al., 2012), we estimated the CD4+ to CD8+ ratio in the Naumova data set. The results showed that the adopted children in this study looked very similar in CD4/CD8 ratios to both family- and orphanage-reared poor children in Russia, and all three groups appeared somewhat immunosuppressed compared to nonadopted children conceived, born, and reared in well-resourced families in the United States. Thus, the differences between the adopted and nonadopted children in cell types in our sample was not due to institutional care, per se, but would also be observed in other poor children from Russia/Eastern Europe. We should also note, of course, that these differences could also be due to allelic differences between Russian/Eastern European populations and the Americans of European descent (Chami & Lettre, 2014). Follow-up studies are needed to replicate these findings through immune phenotyping and functional analyses of how well the immune systems of children adopted from early adverse conditions combat and contain infections.

Regardless of the explanation for the cell-type difference, the presence of such striking differences would have produced highly spurious epigenetic results had we not controlled for them. The need to control for cell type has been noted by others, but is still not routinely done (Houseman et al., 2012; Lam et al., 2012; Liu et al., 2013). Similarly, a previous study showed that because cellular composition changes with age, accounting for cellular heterogeneity is also critical in any study of DNA methylation and age (Jaffe & Irizarry, 2014). As a demonstration of the importance of cell-type correction, we reported 136,339 methylation differences between groups when we did not correct for cell type, and significantly fewer after correcting for cell type. Because type of tissue/cell type is the primary determinant of DNA methylation patterns, the marked differences in immune cell types in the present study would have yielded a vast

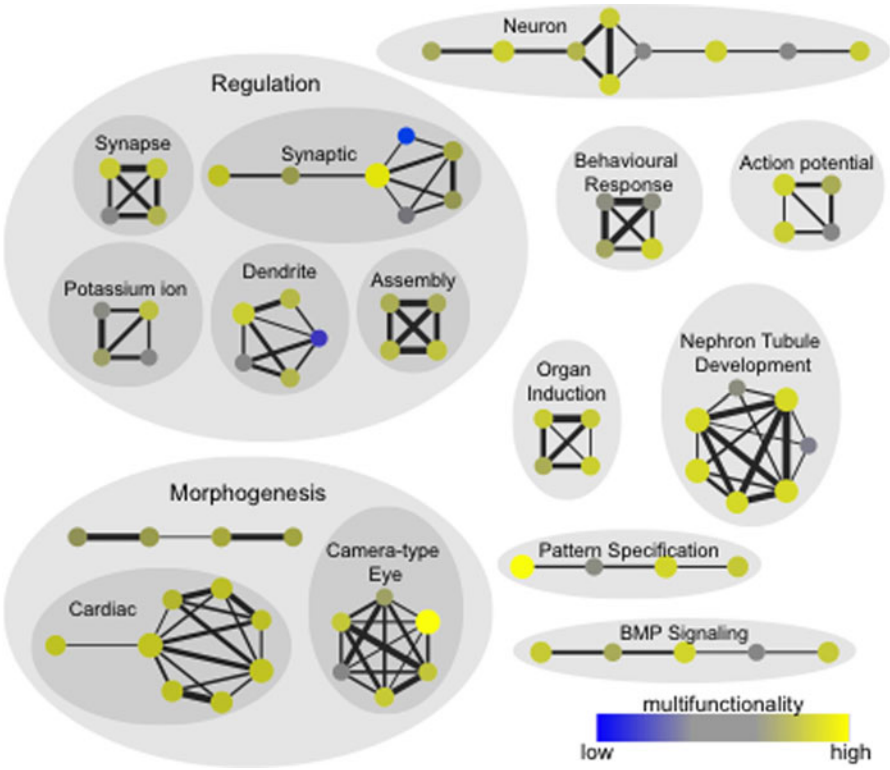

Figure 4. (Color online) Whole-genome epigenome-wide association study found a signature of association between adoption status and DNA methylation, and functional analysis revealed many neurological and developmental processes. Enrichment map for gene ontology (GO) terms significantly enriched in an epigenome-wide association study with a false discovery rate of <0.05 assessed by ErmineJ. Only clusters with four or more GO terms were included in this figure, and cluster names were assigned by WordCloud. GO terms are represented by nodes, node size indicates number of genes in each term, and node color indicates multifunctionality score for each term. Edge width indicates number of shared genes between terms.

overestimation of methylation differences between groups. Examining cell type and then correcting for it in the present study was clearly vital to determining the true signals of DNA methylation associated with the early life differences between the adopted and nonadopted groups.

After correcting for cell type, none of the specific sites survived correction for multiple testing when we used all of the probes on the array. However, the *p* values were clearly skewed, indicating that there was a signal present that did not reach statistical significance. We addressed this problem in two ways. First, we adjusted the correction factor by removing all the methylation sites that did not show any variation. This data reduction approach is modeled after published studies aimed at reconciling the large number of data points obtaining in genomic DNA methylation or gene expression studies with generally small cohort sizes (Bourgon et al., 2010; Teh et al., 2014). After taking the invariant probes out, we noted 19 genes mapping to the 30 sites that survived correction. These were a varied assortment (Table 1). Transmembrane protein 200C, which had the greatest number of sites that were differentially methylated between groups, is a gene with relatively little known functional information. It was identified as a candidate for genes related to psychiatric illness associated with a pericentric inversion of Chromosome 18 (Pickard et al., 2005), but its function has not been elucidated. *PPP1R3G*, which had three differentially methylated CpGs, is involved in the regulation of glucose homeostasis in the fasting-feeding cycles and the rise in glucose following a meal (Luo et al., 2011; Zhang et al., 2014). Given that growth delay is associated with early life adversity in impoverished children (Fernald & Grantham-McGregor, 2002) and children in institutional care (Johnson & Gunnar, 2011), perhaps alterations in a gene involved in regulating energy metabolism in relation to food intake could be expected. *GLYATL2*, with two differentially methylated sites, is a glycine acetyltransferase that is also relatively uncharacterized. It is part of the family of glycine-*N*-acyltransferase whose biological activities include antinociceptive, anti-inflammatory, and antiproliferative effects (Waluk, Schultz, & Hunt, 2010). *miR-324* has been implicated in both immune function upon infection and as an animal model of posttraumatic stress disorder (Balakathiresan et al., 2014; Chang et al., 2014). This might reflect the observed differences in immune status of the adopted children and bears further scrutiny. Finally, *CYP1A1*, also with two differentially methylated CpGs, is a highly multifunctional metabolic enzyme, and its differential methylation related to early life adversity may be one of the reasons for the high number of terms in the GO analysis. It

is interesting that an increase in DNA methylation of *CYP1A1* has been reported in response to exposure to cigarette smoke in early life (Lee et al., 2014). We found higher DNA methylation in the adopted children, which could indicate that they experienced higher exposure to cigarette smoke both before and after birth, consistent with the high rate of smoking in these children's countries of origin. We supported this finding by examining a subset of CpGs in the *AHRR* gene that has been associated with cigarette smoke, and although AHRR was not differentially methylated in our full or filtered data set, the pattern observed when we examined it specifically was consistent with increased exposure in the adopted children. This hypothesis was further supported using a subset of CpGs in the *AHRR* gene that has been associated with cigarette smoke. Note that exposure to cigarette smoke would not be specific to being institutionally versus family reared. Of course, this difference could be due to the youth taking up smoking themselves. We did not ask about whether the participants were smoking. We did ask the parents to report on externalizing problems, which is associated with teen smoking and did differ by group. However, when we included externalizing as a covariate in the *AHRR* gene analysis, the methylation difference was still noted.

Second, we performed a functional enrichment analysis using ErmineJ's Gene Score Resampling method. We chose this method because it reduces bias by using a rank-ordered list of genes and *p* values to identify GO terms, and thus is ideal for examining patterns in the larger data set encompassing all CpG sites (Gillis et al., 2010). Because it does not use a *p* value significance threshold, it was appropriate for this analysis and served as its own background. Many of the high-ranked genes in the resulting list of differentially methylated sites had many GO terms associated with them due to multifunctionality. After reducing this effect by performing clustering analysis, the results pointed to two types of genes differentially methylated between groups. Specifically, neuronal and developmental gene clusters resulted, suggesting wide-ranging effects of early life histories.

Many of the functions associated with highly ranked genes in the genome-wide analysis reflected known effects of early life adversity. For example, alteration in renal development is one of the mechanisms linking maternal stress and nutrition to offspring late-life metabolic syndrome and cardiovascular disease (Barker, 1997). Thus, it was noteworthy that one of the clusters from the functional analysis was nephron tubule development. Similarly, bone morphogenic proteins signaling may be of particular relevance because of the marked bone growth delay observed among infants and toddlers growing up in institutions (Johnson & Gunnar, 2011). Certainly, given the numerous differences in cognition and brain structure (Sheridan et al., 2012; Tottenham et al., 2010), it is no surprise that two of the clusters relate to the nervous system and its regulation, though it is unclear how our observations in blood reflect changes in brain. It has been shown that concordance between blood and brain is variable between CpGs sites, with some showing greater similarity than others (Davies et al., 2012; Farré et al., 2015). In addition, the many genes with multiple functions related to morphogenesis of the eye and heart may be consistent with vision problems frequently noted among children reared in institutions (Eckerle et al., 2014) and may suggest the potential for cardiovascular disease as these children age.

There were a number of limitations to the current study. First, with a goal of examining the long-term impacts of early adversity when it is followed by rearing under low-risk conditions, to rule out ethnicity as the key factor, the ideal comparison group would have been nonadopted Russian/Eastern European children reared in families of comparable educations and incomes to the adoptive American families. To isolate institutional care as the factor producing the long-term outcomes following removal from early adversity, one would have compared poor Russian/Eastern European children who were and were not institutionalized, as Naumova et al. (2011) did, but then who were all removed from conditions of adversity by around 2 years of age and placed in low-risk, high-resourced families until they were adolescents. Studies like the Bucharest Early Intervention Study (e.g., Drury et al., 2012) are fairly well set up to conduct the appropriate study, and it is hoped that they will be able to replicate some of the present findings. In the present study, we are not attributing the effects to institutional care, however, but rather we are assuming that everything that differed between the groups from conception until adoption may have contributed to the differences we noted. Children who are abandoned or removed from their families and placed in institutional care often have difficult prenatal histories and/or are removed from their families because of neglect, abuse, and/or parental incarceration (Gunnar et al., 2000). Thus, we are examining epigenetic differences between children who experienced significant early life stress and exposure to different environmental factors, such as smoke, compared to those whose early development occurred in a relatively low-stress context.

Second, because we did not have blood collected at adoption to compare with our findings, we cannot determine whether methylation differences during adolescence represented a change from those that would have been noted before the children spent time in their families. Longitudinal work is clearly needed. Third, the effect sizes are modest, albeit consistent with other similar work in the literature (Mehta et al., 2013; Naumova et al., 2011). Fourth, the sample size was limited, and more participants would have provided power to detect differences in methylation at more sites even after *p*-value corrections. Fifth, a differential complete blood count was not conducted on the original blood samples, so cell types were calculated based on a published algorithm, and effect of cell type was regressed out of the data (Houseman et al., 2012; Jones et al., 2015). Because both the algorithm and the regression method have been extensively used, and our PCA analysis indicates that cell-type effects were efficiently removed from our data set, we are confident in both methods. Nonetheless, it is possible that vestiges of cell-type effect remain present in the data, or that important DNA methylation changes that

were present only in a single cell type are being removed by our correction method. Sixth and finally, while the adopted and comparison youth were Caucasian, most of the nonadopted youth were of Nordic and European descent, while the adopted youth were of Russian/Eastern European heritage. Thus, allelic differences between the groups may have influenced the results.

Despite these limitations, the results raise the possibility of differences in DNA methylation patterns as a function of early adversity that persist for years following removal from adversity and placement in supportive, well-resourced homes. However, because of the limitations noted, these findings should be considered preliminary and in need of replication.

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