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DNA methylation at stress-related genes is associated with exposure to early life institutionalization

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Abstract

Objectives—Differences in DNA methylation have been associated with early life adversity, suggesting that alterations in methylation function as one pathway through which adverse early environments are biologically embedded. This study examined associations between exposure to institutional care, quantified as the percent time in institutional care at specified follow-up assessment ages, and DNA methylation status in two stress-related genes: *FKBP5* and *SLC6A4.*

Materials and Methods—We analyzed data from the Bucharest Early Intervention Project, which is a prospective study in which children reared in institutional settings were randomly assigned (mean age 22 months) to either newly created foster care or care as usual (to remain in their current placement) and prospectively followed. A group of children from the same geographic area, with no history of institutionalized caregiving, were also recruited. DNA methylation status was determined in DNA extracted from buccal epithelial cells of children at age 12.

Results—An inverse association was identified such that more time spent in institutional care was associated with lower DNA methylation at specific CpG sites within both genes.

Discussion—These results suggest a lasting impact of early severe social deprivation on methylation patterns in these genes, and contribute to a growing literature linking early adversity and epigenetic variation in children.

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Keywords

epigenetics; early life adversity; stress

Exposure to adverse early life experiences can have deleterious effects on child development and functioning (Bateson et al. 2004), as well as a broad range of long term physical and mental health consequences, including cardiovascular disease (Galobardes et al. 2006), depression, and anxiety (Phillips et al. 2005). A number of biological pathways are suggested mediators linking adverse early experiences and poor health and development. Recent studies have documented associations between early adversity with biological markers including altered cortisol regulation (Struber et al. 2014), cytokines (Slopen et al. 2012), and telomeres, a cellular indicator of stress and aging (Drury et al. 2012b).

In addition to these pathways, DNA methylation is an epigenetic mechanism that has been associated with early life adversity and may facilitate the biological embedding of early life experience (Foley et al. 2009; Hertzman 1999; Kuzawa and Sweet 2009). DNA methylation usually refers to the methylation of cytosine guanine dinucleotides (i.e., CpG sites) in the DNA. Alterations in DNA methylation have been associated with adverse early caregiving environments across several species. One example is that low licking and grooming behavior by rat mothers in the first week of life has been associated with increased DNA methylation in the hippocampus of the offspring in the promoter region of *NR3C1*, a gene that encodes the glucocorticoid receptor (Weaver et al. 2004), and at the *GAD1* promoter, a gene that influences the development of the GABA system (Zhang et al. 2010). There is now extensive evidence in both animals and humans linking early life stress with altered DNA methylation across various brain regions and in peripheral tissues across many stress-response related genes (Jawahar et al. 2015; Turecki and Meaney 2014).

One gene with established associations with maternal care, early life adversity, and long-term child developmental outcomes is *SLC6A4*, the gene that encodes the serotonin transporter (5-HTT). Serotonin (5-HT) is involved in the development of the central nervous system, including multiple pathways that have been implicated in neuropsychiatric disorders (Jans et al. 2007). The 5HTT is a key regulator of 5HT levels and a substantial body of literature has examined genetic and stress-related alterations in *SLC6A4* in association with early life adversity and long-term health outcomes (Dayer 2014). For example, polymorphic variants and altered DNA methylation at *SLC6A4* may moderate an individual's response to adversity and contribute to altered cortisol stress responses (Buchmann et al. 2014; Ouellet-Morin et al. 2013), and the development of psychopathology (Bogdan et al. 2014; Karg et al. 2011). Both adverse prenatal and early childhood exposures have been linked to altered DNA methylation of *SLC6A4* in animal models and humans. For example, in macaques, early maternal separation has been associated with increased methylation of *SLC6A4* in blood cells, lower 5HTT expression, and increased stress reactivity (Kinnally et al. 2010). In humans, studies have similarly shown increased methylation in response to early life stress; e.g. higher levels of *SLC6A4* methylation have been found in adults exposed to stressful or traumatic events in childhood or adolescence (Beach et al. 2010; Beach et al. 2011; Duman and Canli 2015; van der Knaap et al. 2015). Additionally, a positive association has been

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identified between early and recent life stress with increased DNA methylation and altered gene expression in adult men (ages 18–77), in interaction with a polymorphism of *SLC6A4* (Duman and Canli 2015). In contrast to these studies, increased maternal depressed mood during the 2nd trimester of pregnancy was associated with *decreased* methylation in the promoter region of *SLC6A4* in both maternal and neonatal whole blood samples (Devlin et al. 2010). Taken together, these studies indicate that adverse prenatal and early life exposures have been associated with both increased and decreased DNA methylation of *SLC6A4*, and altered expression of 5HTT.

FK506 Binding protein 5 (FKBP5) is a member of the glucocorticoid receptor complex, which facilitates termination of the cortisol stress response by altering the sensitivity to glucocorticoid receptor negative feedback (Binder 2009). Specifically, elevated FKBP5 gene expression reduces the ability of the GR complex to translocate to the nucleus, decreasing transcriptional activation, and subsequently down regulating expression changes initially triggered by elevated glucocorticoids. Similar to that seen with *SCL6A4,* alterations in *FKBP5* methylation, gene expression, and cortisol levels, have been reported in relation to adverse early life exposures. Lower DNA methylation at intron 7 of *FKBP5* was found in the blood of adults exposed to childhood trauma relative to those unexposed, in those carrying a risk allele at this gene (Klengel et al. 2013). Similarly, in a study of young impoverished children (aged 3–5), those who experienced maltreatment showed significantly lower methylation at two CpG sites in this same gene region (Tyrka et al. 2015). In contrast, *increased* DNA methylation at intron 7 has been observed at multiple CpG sites in *FKBP5* in Holocaust survivors, although lower methylation was observed in their offspring, suggesting an intergenerational biological accommodation of trauma at this gene (Yehuda et al. 2015). DNA methylation at sites outside of intron 7 were also associated, in a genotype dependent manner, with a history of physical and sexual abuse in the offspring of Holocaust survivors; methylation levels in this gene were also associated with waking cortisol levels (Yehuda et al. 2015).

Institutional care represents an extreme form of early life adversity characterized by low-quality caregiving with little individual attention that has been associated with lasting negative effects on social-emotional development, mental health, cognitive function, and neural development (Fox et al. 2011; McLaughlin et al. 2011; Nelson et al. 2007; Nelson et al. 2014; Pollak et al. 2010). Physiologically, institutional care has been linked to blunted cortisol reactivity and a less steep diurnal cortisol decline, when compared to children who entered foster care at an earlier age or were adopted (Koss et al. 2014; McLaughlin et al. 2015). While some deficits have been found to be mitigated by removal from institutional care, the cumulative exposure to institutional rearing, as well as the age at which a child is removed from institutional care, have been found to significantly moderate recovery (Nelson et al. 2014).

To our knowledge, only one study to date has investigated associations between institutional care and DNA methylation (Naumova et al. 2012). This study assayed DNA methylation in lymphocytes across 27,000 CpG sites in 14 individuals in institutional care compared to 14 raised by biological parents. They identified small but significant differences (mostly increased methylation) at 914 sites between the two groups of children. Although intriguing,

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the limited sample size, wide variation in exposure to institutional care, and limitations of the selected 27k Illumina microarray which only examines, on average, 1–2 CpG sites per gene, indicate that replication and examination of gene-specific methylation in larger samples sizes is needed.

The Bucharest Early Intervention Project (BEIP) is the only randomized controlled trial of foster care compared to care as usual for children abandoned and placed in institutions at birth or early in life. This ongoing longitudinal study began when children were on average 22 months of age. Children living for more than half of their lives in one of six orphanages for young children in Bucharest were randomized to a newly created foster care program or to continued institutional care. A group of children without exposure to institutional care were recruited from the same maternity hospitals at baseline as the children raised in institutions (Zeanah et al. 2003) and from the same public schools attended by institution reared children at the age 8 assessment (Fox et al. 2011). The randomized design, with variable amount of cumulative exposure to institutional care, provides a unique opportunity for the exploration of the association between a well-characterized exposure to severe psychosocial deprivation and DNA methylation. Here, we analyzed the associations between the percent of a child's life spent in institutional care and DNA methylation in buccal cells at two well-studied stress-related genes, *SLC6A4* and *FKBP5*. We specifically chose to investigate the promoter region of *SLC6A4* and an enhancer region of *FKBP5*, because of the extant literature indicating altered methylation at these sites as a consequence of negative early adverse experiences as well as their established linked to cortisol function (Devlin et al. 2010; Klengel et al. 2013), specifically found to be altered in this cohort (McLaughlin et al. 2015). Based on these studies, we hypothesized that DNA methylation would vary based on the cumulative exposure an individual child had to institutional caregiving. Further, we hypothesized that the percentage of time in institutional care measured earlier in development would be a stronger predictor of methylation at age 12 relative to later time points in childhood.

MATERIALS AND METHODS

Study population

The original study sample was 136 children, recruited between the ages of 6 and 31 months, and assessed on a number of measures (for sample details see Nelson et al. 2014; Zeanah et al. 2003). Following baseline assessment of the ever institutionalized group of children (EIG), half of the children (n=68) were randomly assigned to care as usual (Care as Usual Group; CAUG), and the other half (n=68) were placed in high-quality foster care (Foster Care Group; FCG). Children have been prospectively followed and assessed with a range of biological, physical and social-emotional and cognitive measures at 30, 42, and 54 months and 8 and 12 years of age. An additional group of 72 children who had never spent time in an institution (Never Institutionalized Group; NIG) were recruited from the same maternity hospitals as the institutionalized cohort as well as area elementary schools (Nelson et al. 2014). Following randomization into foster care, the local child protection authorities in Bucharest made all decisions regarding placement of the children, in accordance with Romanian law. Details of the foster care program created for this project, the follow-up, and

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ethical considerations are described elsewhere (Nelson et al. 2014; Smyke et al. 2010; Zeanah et al. 2012). As a result of the BEIP study design, there was significant variation in the total amount of exposure to institutional care. The current assessment was conducted on DNA extracted from buccal samples collected at age 12 from 127 individuals. Among these, 121 samples had sufficient DNA for methylation assays, and 117 samples produced reliable DNA methylation data (112 for *SLC6A4* from 106 for *FKBP5*). The final set of analyzed samples came from 82 children who had ever spent time in an institution and 35 children who had never been institutionalized.

The study protocol was approved by the University Institutional Review Boards (IRBs) of Boston Children's Hospital, the University of Maryland, Tulane University, University of Bucharest, and Vanderbilt University. As dictated by Romanian law, consent was given by the local Commission on Child Protection for each child participant who lived in their sector of Bucharest.

Measures

Percent time in institutional care—Detailed life history data has been collected longitudinally since study initiation. A measure of percent time spent in institutional care was calculated based on the cumulative number of days the child has resided in institutional care. This measure was calculated at each assessment time point for each child individually. Thus, the measure referenced throughout the paper as "percent time in institutional care" represents the percent of a child's life up to a particular age that the child spent in institutional care. Due to the range of age at placement into foster care and variation in the age at which children initially entered institutional care, this variable is most reflective of cumulative exposure to institutional rearing. This measure was calculated at baseline, 30, 42, and 54 months, as well as 8 and 12 years. Percent time in institutional care for the never institutionalized children was set at 0 for all time points.

DNA methylation—Genomic DNA was extracted from buccal swabs collected from children at age 12 using standard protocols (Qiagen, Valencia, CA). The level of DNA methylation was assessed via pyrosequencing at 6 CpG sites within the promoter region of the *SLC6A4* gene and at 2 CpG sites within intron 7 of the *FKBP5* gene. All CpG sites were located within or very near to transcription factor binding sites, as annotated by Encode chip-seq experiments in the UCSC Genome Browser. See Supp. Figure 1 for locations of CpG sites assayed and their relation to transcription factor binding sites in each gene.

In brief, 150ng of DNA from each sample was bisulfite converted in duplicate using the EZ DNA Methylation Gold Kit (Zymo Research, CA), according to manufacturer's protocol. Bisulfite-converted DNA was mixed with 0.2uM of each primer and amplified using the HotstarTaq plus Master Mix (Qiagen, CA). A bisulfite conversion check was included in each assay to verify full conversion of the DNA. Methylation levels for all CpG sites were assessed using the Pyromark Q24 pyrosequencer. The assay was validated with a methylation scale (0%, 20%, 40%, 60%, >80%) in duplicate created from whole genome amplified DNA (representing 0% methylation), and DNA treated with CpG methyltransferase *M.SssI* (representing >80% methylation). For each sample, PCRs were

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performed on each of the duplicate bisulfite treatments using the following protocol for both genes: one cycle of 95°C for 5 min, 45 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and 72°C for 10 min. Primer information for each gene is provided in Supp. Table 1. If the difference between two replicates exceeded two standard deviations (SDs) of the variation in the entire study population, a third bisulfite treatment was tested and the average of the two closest results was used. After a third bisulfite treatment was conducted on 8 samples, the SD for all the technical replicates for *SLC6A4* ranged from 1.1 to 3.1 across the 6 tested sites, and from 4.8 to 5.9 for the two tested sites in *FKBP5*. The mean difference between technical replicates in this study was 2–3% across both genes. Of the 121 samples assayed for methylation, 4 samples failed to amplify for either gene. Lab technicians were blind to sample group assignment (i.e., FCG, CAUG, NIG).

Covariates—Additional variables controlled for in the regression models included age at time of buccal swab collection, sex, ethnicity (Romanian versus other) as reported by Romanian research staff involved in the study, and pubertal status as measured by Tanner Stage quintiles at age 12 (Marshall and Tanner 1969; Marshall and Tanner 1970).

Statistical Analyses—Methylation scores were examined and outliers were excluded for specific sites (ranging from 0–3 excluded per each site), as determined by scores that fell beyond three standard deviations from the mean. Next, bivariate crude correlations were analyzed between each CpG site in each gene with the percent time in institutional care. Given multiple comparisons, we adjusted for the number of sites tested within each gene using a Bonferroni multiple testing correction (e.g., α=.05/6; p=.008 for *SLC6A4*; α=.05/2; p=.025 for *FKBP5*). For bivariate associations that remained significant following this adjustment, a series of hierarchical linear regression analyses, adjusting for demographic variables (i.e., age, sex, ethnicity, and pubertal stage), were then conducted. Demographic variables were included in Step 1, and percent time in institutional care was additionally included in Step 2, in the prediction of DNA methylation level. All methylation sites were examined for normality and a visual inspection found each to be within normal limits. In addition, regression residuals were plotted and visually inspected and were normally distributed.

In sensitivity analyses, children who had never spent time in an institution (NIG) were excluded and only those in the EIG (n=82) were analyzed in relation to percent time in institutional care. Pairwise correlations and linear regressions including the same covariates as in the primary analyses were conducted on this subset of the sample in the same manner as in the total sample.

RESULTS

Population characteristics

Sample characteristics across the EIG and NIG groups of children are presented in Table 1. The mean age at which the DNA was collected for methylation analyses in the total sample was 12.5 (*SD*=0.4) years, 51% of the sample was male, and 63% identified as Romanian in ethnicity, with the remainder identified as Rroma (27%) or other (9%). More of the children

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in the EIG relative to NIG were identified as Rroma, but no other demographic differences between groups was detected.

Associations between institutionalization history and DNA methylation at *SLC6A4*

Percent time in institutional care, measured at each time point, was found to be significantly correlated with DNA methylation at two CpG sites out of six tested in the promoter region of *SLC6A4* (Table 2). Specifically, CpG5 was negatively correlated with percent time in institutional care at five of the six time points, and CpG6 was negatively correlated with percent time in institutional care across all measured time points. After Bonferroni correction for all CpG sites tested within this gene (threshold at p=.008), Cpg5 remained significantly associated with percent time in institutional care at 30 and 42 months; CpG6 remained significantly associated with percent time in institutional care at 30, 42, and 54 months.

Associations between institutionalization history and DNA methylation at *FKBP5*

A significant negative correlation was found between CpG1 in *FKBP5* with percent time in institutional care measured at baseline (Table 2), though this association did not remain significant after adjusting for Bonferroni correction (threshold at p=.025).

Associations within ever institutionalized youth between institutionalization amount and DNA methylation at *SLC6A4* and *FKBP5*

In order to determine if the relative amount of exposure to institutional caregiving was associated with DNA methylation, we subsequently conducted correlation analyses within the ever institutionalized youth only (Table 3). For *SLC6A4*, methylation at CpG5 and CpG6 were both negatively correlated with the percent time in institutional care at 30 months, while methylation at CpG6 was also negatively correlated with the percent time in institutional care at 42 months. After Bonferroni correction for all CpG sites tested within this gene, methylation at both CpG5 and CpG6 remained significantly correlated with percent time in institutional care at 30 months. For *FKBP5*, a significant negative correlation was found between methylation at CpG1 with percent time in institutional care at baseline, which remained significant after adjusting for Bonferroni correction.

Hierarchical linear regressions

Regressions within the total sample—Next, we examined whether the associations in the total sample that were significant after multiple correction adjustment remained significant with the inclusion of demographic covariates. Regressions using the total sample were conducted only for CpG sites within *SLC6A4*, as no correlations with sites in *FKBP5* passed the multiple testing correction. Specifically, age at DNA collection, sex, ethnicity, and pubertal stage were examined along with percent time in institutional care in relation to DNA methylation at CpG sites 5 and 6 of *SLC6A4* (Table 4). In the model including only the demographic covariates, CpG5 methylation was significantly positively associated with age ($t(100)=2.19$, p=.031, β=.21), and marginally negatively associated with pubertal stage ($t(100)=-1.87$, p=.065, β=−.19). The association of sex or ethnicity with methylation was not significant. Importantly, the negative association between percent time in institutional

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care and DNA methylation at CpG5 remained significant following adjustment for covariates. In the model including demographic covariates for CpG6, there was no significant association between any of the covariates and methylation. Again, the negative association between percent time in institutional care at all time points and DNA methylation at CpG6 remained significant following adjustment for covariates. While the effect size for each 1% increase in time spent in institutional care was very small at both sites of *SLC6A4* (1–2% lower methylation), this effect would be amplified when considering longer periods of time spent in institutional care. For example, a child with a 10% increase in total institutional care, (i.e. 1.2 additional years in care), would predict a 10–20% decrease in methylation. Further, when added to the regression models, time spent in institutional care at 30 months predicted an additional 9% and 11% of the variance in methylation, respectively for CpG sites 5 and 6.

Regression analyses within ever institutionalized youth only—In order to determine if the amount of time spent institutionalized affects the results within only the EIG, we conducted the regression analyses for methylation at *SLC6A4* and *FKBP5* in EIG only (Table 5). With the smaller sample, there was a reduction in the number of significant associations found between percent time in institutional care and methylation for *SLC6A4*. Percent time in institutional care at 30 months, but not at other time points, remained a significant predictor of reduced methylation at both CpG5 and CpG6 of *SLC6A4*. In addition, the association between percent time in institutional care at baseline and CpG1 in *FKBP5* was significant among institutionalized youth only. The relationship between institutional care and methylation level at these CpG sites is presented in Figure 1. In the model of methylation at *SLC6A4* with just demographic covariates included, only pubertal stage was significantly associated with methylation at CpG5 ($t(66)=-2.20$, $p=.031$, $\beta=-.27$), whereas no covariates were significantly associated with methylation at CpG6. In the model for *FKBP5* with just demographic covariates included, pubertal stage was marginally associated with methylation at CpG1 ($t(62)=-1.95$, $p=.056$, $\beta=-.24$).

DISCUSSION

This is the first study, to our knowledge, to demonstrate the lasting impact of exposure to early institutional care on DNA methylation in two genes related to the stress response systems- *SLC6A4* and *FKBP5*. In both genes, increased percent time spent in institutional care across early development was associated with lower DNA methylation at age 12. These associations remained significant after adjusting for covariates of age, sex, ethnicity, and pubertal stage. These results are consistent with other findings at age 12 in these youth including alterations of cortisol reactivity (McLaughlin et al. 2015), and increased risk in psychopathology (Humphreys et al. 2015), suggesting a lasting cross-domain impact of institutional rearing that begins at the cellular level and is also found in physiologic and behavioral outcomes. Our findings of persistent changes in methylation in key regulatory regions of genes associated with the HPA axis and psychopathology following adverse early life experiences suggest that altered methylation is reflective of similar biological pathways and that together, they contribute to the elevated and persistent risk for psychopathology within these individuals.

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Timing of exposure

The longitudinal design of the BEIP study enables the unique examination of whether developmental differences exist in relation to the amount of institutional care and methylation at age 12. For *FKBP5,* the strongest associations were found with percent time in institutional care at baseline, when children were on average 22 months of age. The importance of early rather than later exposure to institutional rearing for *FKBP5* is generally consistent with prior studies of this gene, where an association was found between methylation at *FKBP5* and child abuse, but not later life trauma (Klengel et al. 2013). In contrast, the strongest associations with *SLC6A4* were at later the time points 30, 42, 54 months, and 8 years, a finding somewhat consistent with our previous findings in which 5httlpr genotype was a significant predictor of disinhibited social behavior at 30 months of age and later (Drury et al 2012). Other studies of methylation in this gene also found timing-specific effects; e.g. Devlin et al (2010) reported decreased methylation in the same region specifically at the 2nd trimester of pregnancy, but not at earlier or later periods of pregnancy.

The different patterns of associations between the two genes tested suggest important developmental differences and hint at the possibility of gene-specific sensitive periods during which environmental exposures, such as caregiving, may have important lasting effects on DNA methylation. This finding of varied timing of effects across the genes is consistent with previous genetic studies with the BEIP where differential associations with genetic variants and outcomes were found for *BDNF* and *SLC6A4* (Drury et al. 2012a). In that study, the impact of a genotype within *SLC6A4* was greatest between baseline and 42 months of age, while the impact of a genotype in *BDNF* was more apparent at later time points. A significant timing effect has also been identified for FKBP5 mRNA expression in the prefrontal cortex, which has been shown to increase expression in adolescence and peak in adulthood, suggesting that childhood and adolescence may be particularly vulnerable periods for stress exposures, before *FKBP5* may be able to fully regulate the activity of the glucocorticoid receptor (Weickert et al. 2016). Longitudinal studies, ideally with DNA methylation, genotype, and gene expression levels, measured at multiple time points, are needed to determine the specific role of each gene within each developmental period, and how methylation and expression patterns may change across time both in response to different environmental exposures and developmentally.

Direction and magnitude of effects

The small magnitude of the effect of institutionalization on methylation identified in both genes is similar to that reported in some prior studies, though the direction of the effect varies across studies. Specifically, one study examined DNA methylation at other CpG sites within *FKBP5* in relation to prenatal stress exposures, such as chronic stress and war trauma in the Congo (Kertes et al. 2016). This study found higher methylation at one CpG site (cg03546163) in *FKBP5* in placenta and cord blood. While this was the opposite direction of the effect identified in our study, this difference could be explained by the fact that they were examining a different CpG site in different tissues. In other research, the direction of effect was consistent with our study, such that early childhood trauma was found to be associated with an average of 12.5% *lower* DNA methylation in blood cells across three CpG sites in intron 7 of *FKBP5*, two of which were the same sites assayed in this study (Klengel et al.

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2013). Interestingly, genotype appeared to moderate this effect in their study such that individuals who were carriers of the A allele (rs1360780) and exposed to childhood trauma had lower methylation than those without the A allele with or without childhood trauma. Because of these findings, we subsequently tested whether *FKBP5* genotype moderated our findings. However we did not detect any effect moderation by genotype (data not shown). While our sample represented a typical distribution of alleles (GG, n=71; GA, n=33; AA, n=9) relative to other European populations (dbSNP 2016), it is possible we were unable to detect this interaction effect because of our small sample size.

For *SLC6A4*, prior studies have also shown conflicting directions of effect in response to childhood adversity. Decreased methylation was found in this same gene region in newborn children exposed to prenatal maternal depression, consistent with our current findings (Devlin et al. 2010). However, in a separate study, 10-year old children exposed to bullying were found to have *increased* buccal cell methylation (Ouellet-Morin et al. 2013). These differences in direction of effect between studies could be due to differences in the developmental stage at which methylation was determined, duration, or nature of exposures, and the source tissue of DNA used for methylation analyses (Lupien et al. 2009).

Our results differ somewhat from the findings of the only previous study of methylation in children with a history of institutional care (Naumova et al. 2012). In their study, genome-wide analysis did not detect significant differences in DNA methylation obtained from peripheral blood at either *FKBP5* or *SLC6A4* in institutionalized children. However the 27K Illumina array only assays two sites in each of these genes, neither of which match the specific sites assayed in this study. In the study by Naumova and colleagues, a large number of sites throughout the genome were identified with differential methylation, the majority of which indicated increased methylation in children with a history of institutionalized caregiving. However, 11% of the sites demonstrated hypomethylation in institutionalized children suggesting that the relationship between early negative caregiving environments and methylation is unlikely to be unidirectional across the genome: some genes may be hypomethylated while others will be hypermethylated, a pattern consistent with primate studies (Provencal et al. 2012).

Potential implications for expression and health

Given the small magnitude of methylation differences we detected, and the complexity of relationship between methylation and expression, we cannot infer how these differences will impact lifetime health. It is important to note that the CpG sites demonstrating significant differences were located within transcription factor binding sites, suggesting a likely role in affecting gene expression levels. Specifically, CpG 5 and 6 in *SLC6A4* are located within the binding site for the transcription factor EZH2 and CpG 1 and 2 in *FKBP5* are located within the binding site for the transcription factor NR3C1 (Supp Figure 1). Furthermore, the two sites assayed in *FKBP5* lie within a glucocorticoid response element within an enhancer region in intron 7, which loops around to directly interact with the transcription start site of this gene (Klengel et al. 2013). Larger sample sizes are needed to definitively test causal pathways of methylation on downstream physiologic and behavioral outcomes. The effect of small changes in single genes on complex physiologic processes and developmental

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psychopathology is expected to be small. As such, repeated measures with longitudinal data are likely required to detect the direct functional impact, particularly in studies such as the BEIP where a significant number of biological and behavioral differences have already been identified as a consequence of early experiences. It is of interest to note that an earlier study of the BEIP demonstrated that children who remained in institutional care exhibited blunted cortisol responses to psychosocial stress compared with children randomized to foster care (McLaughlin et al. 2015). In a similar study, children adopted from institutions or foster care exhibited a less steep diurnal cortisol pattern compared to non-adopted peers, and this blunted diurnal pattern was associated with increased behavioral problems two years after adoption (Koss et al. 2014). While we cannot link the DNA methylation differences in this study to cortisol differences directly, as these effects are likely a result of more than just methylation changes at single sites, taken together these studies support the hypothesis that institutional care results in functional differences in stress response systems that are persistent and likely linked to epigenetic alterations in the genes known to regulate them. Studies that combine methylation, gene expression, genotype, and specific physiologic outcomes, such as cortisol reactivity or vagal tone, represent novel future approaches capable of specifically addressing mechanistic pathways.

Strengths and limitations

Several limitations should be noted. First, our study could not assess the role of prenatal exposures or parental care prior to institutionalization. Second, any analysis of DNA methylation in buccal samples cannot necessarily be generalized to other tissues of interest, such as neural tissues, where methylation patterns may differ. Although buccal swabs comprise primarily epithelial cells, derived from neuroectodermal tissue similar to neuronal cells, some amount of white blood cells may also be collected which may or may not have similar methylation pattern within an individual. Further, a recent genome-wide analysis of samples from dozens of different somatic tissue types identified DNA methylation patterns in buccal samples to more closely cluster with patterns in all other tissue types than did blood samples, suggesting buccal cell methylation may be more representative of other tissues, including brain, than methylation obtained from DNA in peripheral blood (Lowe et al. 2013). Unfortunately, sufficient DNA is not available at this time from multiple earlier time points, future studies are needed to address this issue, ideally in studies that examine the trajectory across different peripheral and central sources.. Despite this limitation the consistency of the association with varying measurements of the proportion of time a child spent in institutional care hints at the existence of gene specific sensitive periods. Finally, despite a relatively small sample size, our study is strengthened by the well characterized, prospective measurement of exposure to institutional care.

The strength of this study lies in the uniqueness of the randomized controlled study design, minimizing the risk of confounding by factors that putatively could influence initial placement in institutional care or subsequent placement into foster care. Further, the longitudinal design permits comparison of the portion of life spent institutionalized across different developmental time points, which revealed both a lasting effect of institutionalization on methylation at both genes as well as putative timing-specific effects. Our decision to test for methylation differences at these specific sites was based on the

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existing data demonstrating well-characterized effects on stress-response systems for both genes and interactions across a range of different studies with early life adversity. Particularly with limited subject numbers, targeted gene analyses supported by molecular studies and neuroscience, minimize the potential for false positive findings, an issue particularly relevant to genome wide studies that fail to leverage the substantial existing neurobiologically-informed research related to the impact of early adversity. Future studies that examine methylation in additional candidate genes involved in stress-response systems, such as the gene that encodes the glucocorticoid receptor (*NR3C1*), or the corticotrophin hormone receptors (*CRHR1, CRHR2 would be of interest.* In studies with larger sample sizes, potential interactions between these candidate genes could also be tested, to determine if methylation states across these genes contribute to any long term psychological or biological outcomes.

While evidence is rapidly accumulating that early life social adversity has profound effects across biological systems, more research is needed to determine the molecular pathways through which these changes occur and the mechanisms underlying the persistence of these effects. Our findings demonstrate an epigenetic path through which early adversity influences DNA methylation within regulatory regions of two well-characterized stress-related genes. These findings have implications for less extreme examples of social deprivation, such as adverse and toxic environments outside of institutions, in which altered methylation at these genes may serve as objective biomarkers of exposure and potential indicators of elevated future risk. With further research on functional consequences of these methylation differences, these findings may ultimately help predict lifetime development of psychopathology in children exposed to early adversity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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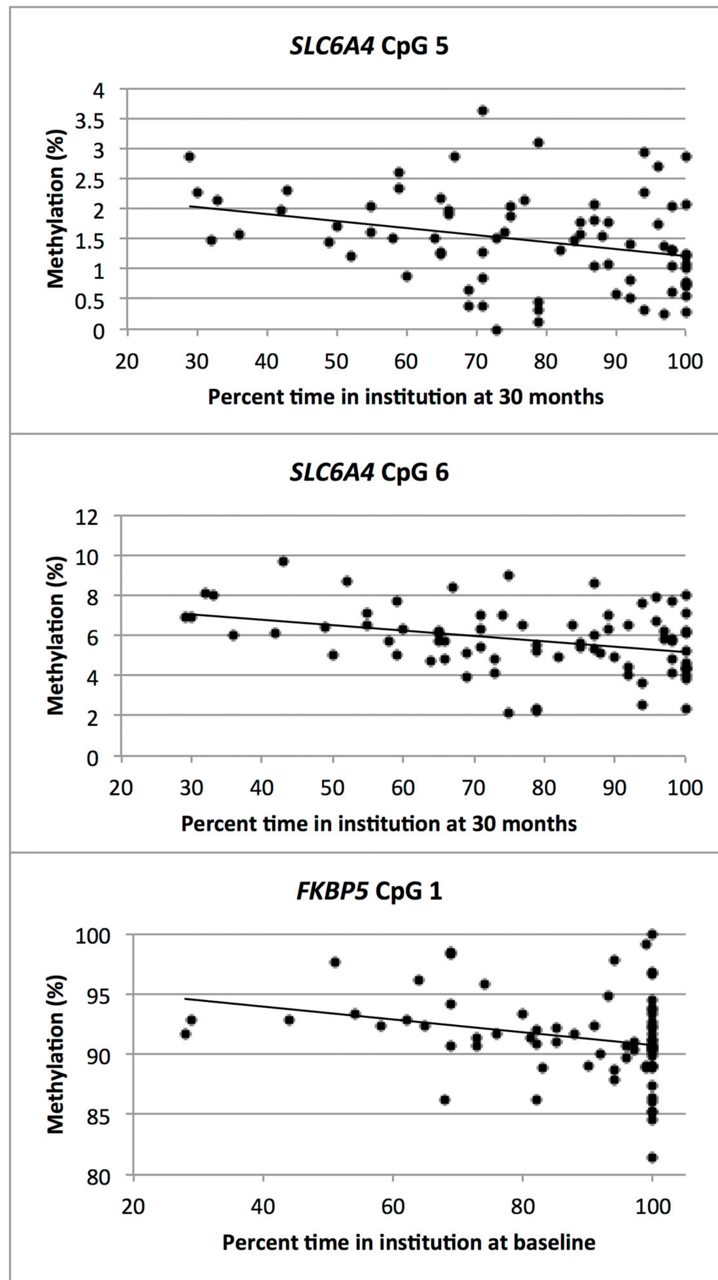

Figure 1.

Relationship between percent time in institutional care and methylation level among children who had ever spent time in the institution (EIG) for CpG sites with significant associations in *SLC6A4* and *FKBP5*.

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Table 1

Sample characteristics across study groups of never and ever institutionalized children.

	Never Institutionalized (n=35)	Ever Institutionalized (n=82)
Sex, n (%)		
Male	17 (49%)	43 (52%)
Female	18 (51%)	39 (48%)
Race/Ethnicity*, n (%)		
Romanian	33 (94%)	41 (50%)
Rroma	2 (6%)	30 (37%)
Other	0 (0%)	11 (13%)
Proportion of time in institutional care*, mean (SD)		
Baseline	0 (0)	0.87 (0.19)
30 months	0 (0)	0.78 (0.20)
42 months	0 (0)	0.65 (0.22)
54 months	0 (0)	0.57 (0.25)
8 years	0 (0)	0.40 (0.26)
12 years	0 (0)	0.32 (0.27)

[*] Indicates significant differences between groups at $p < 0.05$ in chi-square tests. The variable 'proportion of time in institutional care' represents the proportion of a child's life up to a particular age that was spent in institutional care; the mean and SD of this variable across children is shown at each time point.

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Table 2

Correlation matrix for methylation sites and percent time institutionalized in the full sample.

	SLC6A4 Correlations						*FKBP5* Correlations	
Percent time in institutional care	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**CpG1**	**CpG2**
Baseline	−.09	−.08	.07	−.08	−.23*	−.22*	−.21*	−.10
30 months	−.15	−.12	.05	−.10	−.29**	−.30***	−.18†	−.07
42 months	−.15	−.08	.06	−.04	−.25**	−.29**	−.13	−.04
54 months	−.15	−.05	.05	−.01	−.22*	−.26**	−.11	−.03
8 years	−.11	.01	.03	.01	−.19*	−.23*	−.09	.01
12 years	−.13	.01	.05	.01	−.17†	−.22*	−.08	.03
Mean (SD)	3.49 (1.54)	1.48 (0.68)	3.25 (1.23)	2.58 (1.09)	1.60 (0.81)	6.02 (1.71)	91.81 (3.58)	84.11 (5.36)
Range	0.71–7.46	0.28 – 3.67	1.12 – 7.15	0.37 – 6.10	0.00 – 3.64	2.12 – 11.07	81.47 – 100.00	69.70 – 97.50

Note.

† $p < .10$.

* $p < .05$.

** $p < .01$.

*** $p < .001$.

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Table 3

Correlation matrix for methylation sites and percent time institutionalized in the ever institutionalized youth only.

	SLC6A4 Correlations						*FKBP5* Correlations	
Percent time in institutional care	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**CpG1**	**CpG2**
Baseline	.10	.05	.05	−.05	−.10	−.08	−.27*	−.16
30 months	−.09	−.10	.002	−.09	−.30**	−.33**	−.15	−.06
42 months	−.09	.01	.02	.04	−.18	−.27*	−.02	.003
54 months	−.09	.07	.01	.09	−.10	.20†	.01	.02
8 years	−.04	.12	−.01	.09	−.08	−.16	.02	.05
12 years	−.09	.10	.03	.08	−.08	−.16	.01	.08
Mean (SD)	3.36 (1.47)	1.44 (0.64)	3.30 (1.31)	2.53 (1.09)	1.48 (0.79)	5.78 (1.62)	91.46 (3.57)	83.94 (5.88)
Range	0.78–7.46	0.28 – 3.67	1.12 – 7.15	0.83 – 6.10	0.00 – 3.64	2.12 – 9.71	81.47 – 100.00	69.70 – 97.50

Note.

† $p < .10$.

* $p < .05$.

** $p < .01$.

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Table 4

Regression models for association between percent time in institutional care and two DNA methylation sites in *SLC6A4* in the total sample.

	SLC6A4 CpG5				*SLC6A4* CpG6			
	B (SE)	**β**	R^2	R^2	**B (SE)**	**β**	R^2	R^2
Step 1 (demographic covariates)			.09†				.02	
Step 2								
Percent time institutionalized at 30 months	−.01 (.002)	−.35	.18**	.09**	−.02 (.01)	−.39	.12*	.11***
Step 2								
Percent time institutionalized at 42 months	.01 (.003)	−.31	.16*	.07*	−.02 (.01)	−.37	.11*	.10***
Step 2								
Percent time institutionalized at 54 months	--	--	--	--	−.02 (.01)	−.33	.10†	.08***
Step 2								
Percent time institutionalized at 8 years	--	--	--	--	−.02 (.01)	−.27	.08	.06*

Note.

$^{\dagger}p < .10$.

$^{*}p < .05$.

$^{**}p < .01$.

Models shown only for CpG sites that showed significant correlations with percent time in institutional care at each time point. B (SE) represents the unstandardized regression coefficient and standard error (e.g., percent change in methylation with each percent increase in time spent institutionalized), and β is the beta coefficient for standardized models (e.g. the number of standard deviation changes in methylation with each standard deviation increase in percent institutionalization). R^2 represents the proportion of the variation in DNA methylation explained by percent time institutionalized. All models adjusted for age, sex, ethnicity, and pubertal stage.

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Table 5

Regression models for association between percent time in institutional care and DNA methylation in ever institutionalized youth only.

	SLC6A4 CpG5				*SLC6A4* CpG6				*FKBP5* CpG1			
	B (SE)	**β**	R^2	R^2	**B (SE)**	**β**	R^2	R^2	**B (SE)**	**β**	R^2	R^2
Step 1 (demographic covariates)			.08				.05				.06	
Step 2												
Percent time institutionalized at baseline	--	--	--	--	--	--	--	--	−.05 (.02)	−.28	.14†	.07*
Step 2												
Percent time institutionalized at 30 months	−.02 (.01)	−.43	.22**	.14***	−.03 (.01)	−.35	.15†	.10**	--	--	--	--

Note.

* $p < .05$.

** $p < .01$.

*** $p < .001$.

Models shown only for the CpG site that showed significant correlations with percent time in institutional care at each time point. B (SE) represents the unstandardized regression coefficient and standard error (e.g., percent change in methylation with each percent increase in time spent institutionalized), and β is the beta coefficient for standardized models (e.g., the number of standard deviation changes in methylation with each standard deviation increase in percent institutionalization). Change in R^2 represents the proportion of the variation in DNA methylation explained by percent time institutionalized. All models adjusted for age, sex, ethnicity, and pubertal stage.