

Is serotonin transporter genotype associated with epigenetic susceptibility or vulnerability? Examination of the impact of socioeconomic status risk on African American youth

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Abstract

We hypothesized that presence of the short allele in the promoter region of the serotonin transporter would moderate the effect of early cumulative socioeconomic status (SES) risk on epigenetic change among African American youth. Contrasting hypotheses regarding the shape of the interaction effect were generated using vulnerability and susceptibility frameworks and applied to data from a sample of 388 African American youth. Early cumulative SES risk assessed at 11–13 years based on parent report interacted with presence of the short allele to predict differential methylation assessed at age 19. Across multiple tests, a differential susceptibility perspective rather than a diathesis–stress framework best fit the data for genes associated with depression, consistently demonstrating greater epigenetic response to early cumulative SES risk among short allele carriers. A pattern consistent with greater impact among short allele carriers also was observed using all cytosine nucleotide–phosphate–guanine nucleotide sites across the genome that were differentially affected by early cumulative SES risk. We conclude that the short allele is associated with increased responsiveness to early cumulative SES risk among African American youth, leading to epigenetic divergence for depression-related genes in response to exposure to heightened SES risk among short allele carriers in a “for better” or “for worse” pattern.

Poverty and economic hardship have a range of long-term effects on child development (Conger et al., 2002), resulting in physiological effects (Evans, Chen, Miller, & Seeman, 2012) as well as a range of social and family consequences (Maholmes & King, 2012). It is particularly noteworthy, therefore, that African American youth living in the Southern coastal plain are exposed to one of the most economically disadvantaged areas in the United States. As a consequence, substantial socioeconomic status (SES) risk is experienced by a large percentage of youth at developmentally sensitive stages. This places them at risk for having shorter life expectancy (Braveman, Cubbin, Egerter, Williams, & Pamuk, 2010), and a range of health effects across the life span (Starfield, Robertson, & Riley, 2002). Although the mechanism conferring these adverse effects is not yet well understood, it has been proposed that effects may result, in part, from the ability of the early social environment to restructure the functioning

of the human genome, resulting in long-term changes in transcriptional responses to future social stressors and creating differential risk for adverse outcomes (Cole, 2011).

In addition to its practical importance, SES risk also provides a scientifically attractive index, because measurement is based on relatively objective indicators that can be assessed prospectively and this source of stress is often relatively stable across childhood, with robust effects on a range of outcomes. Because of its potential relevance for regulating transcriptional response and its relative ease of measurement, DNA methylation has emerged as a potential mechanism of interest in accounting for Gene \times Environment ($G \times E$) effects. Using animal models, methylation also has been shown to be responsive to experimental manipulation via change in social status (Tung et al., 2012) and parenting behavior (Champagne et al., 2008; Trollope et al., 2011), further suggesting its potential developmental significance. Similarly for humans, evidence is emerging demonstrating that methylation is responsive to experimental manipulation of stress (Unterhahner et al., 2012). Therefore, examination of the impact of genetic variability on epigenetic response to the environment may be facilitated by attention to cumulative SES risk as a stressor and DNA methylation as an index of epigenetic response.

One potential genetic moderator of interest is a variation in the promoter of the serotonin transporter (*5-HTT*) gene (solute carrier family C6, member 4 [*SLC6A4*]) also referred

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to as the *5-HTT* linked polymorphic region (i.e., the *5-HTTLPR*), which has long been viewed as a potential moderator of environmental stress (cf. Caspi, Hairiri, Holmes, Uher, & Moffitt, 2010). Perhaps more than any other candidate genotype, the *5-HTTLPR* has sparked debate and interest regarding the nature of its effects (susceptibility vs. vulnerability; e.g., Belsky & Pluess, 2009), as well as the consistency and replicability of its effects and its mechanism(s) of effect (e.g., Caspi et al., 2010). Of particular interest has been the continuing debate regarding the potential moderating effect of *5-HTTLPR* on life stress in the prediction of depression (Caspi et al., 2003; Risch et al., 2009). Unfortunately, examination of genetic moderation of environmental stress on depression is hampered by several methodological problems, including the likelihood that there are biological mediators of stress effects on depression, such as epigenetic change, that have not been adequately characterized (see Szyf & Bick, 2013). Because such epigenetic mediators might be more directly, and strongly, affected by $G \times E$ effects than is depression, they have the potential to help clarify the nature of $G \times E$ effects. It is of particular interest to examine the *5-HTTLPR* as a potential moderator of the long-term impact of early cumulative SES risk on epigenetic change, particularly epigenetic change in depression-related genes. To the extent that the interaction of variation in the *5-HTTLPR* with early cumulative SES risk can influence epigenetic change in depression-related genes, this provides a plausible biological mechanism of long-term effect on depressive symptoms. If effects are present for other developmentally important gene pathways, this would suggest relevance for a broader range of developmental outcomes.

The Role of *5-HTTLPR* in Response to Early Cumulative SES Risk

SLC6A4 is a key regulator of serotonergic neurotransmission, localized to 17p13. It consists of 14 exons and a single promoter. Variation in the promoter region of the gene (*5-HTTLPR*) results in two main variants, a short and a long allele. The short variant has 12 copies and the long variant has 14 copies of a 22 base pair repeat element. Among African Americans, a nonnegligible portion of the population carries an extra long, or very long, variant that has 16 copies. The short variant is associated with lower serotonin transporter transcription and reduced efficiency of serotonin reuptake, supporting its potential relevance for a range of serotonergic-linked outcomes (Carver, Johnson, & Joormann, 2008; Carver, Johnson, Joormann, LeMoult, & Cuccaro, 2011). Recent gene expression research indicates that the very long variant is *not* associated with reduced expression (Vijayendra et al., 2012), suggesting that contrasting the response of those carrying one or more short alleles to all others is appropriate in an African American sample.

Evidence across multiple species and multiple methods indicates that genetic variation in the serotonin transporter is related to differential responses to stress (Caspi et al., 2010) and

encourages attention to the developmental implications of variation at the *5-HTTLPR* in the context of elevated early cumulative SES risk. The short allele appears to be associated with increased connectivity between the amygdala and other brain regions (Heinz et al., 2005), amplification of response to verbal and nonverbal threats (Isenberg et al., 1999), and enhanced reactivity to punishment cues (Battaglia et al., 2005; Hariri & Holmes, 2006) and observational fear conditioning (Crişan et al., 2009). Because SES-related risks are associated with reduced occupational and educational opportunity, frequent housing adjustments, changes in employment status for parents, as well as chronic exposure to interpersonal and institutional racism (Dressler, Oths, & Gravlee, 2005), greater activation in response to a range of perceived threats could be particularly consequential for youth exposed to elevated early cumulative SES risk. Underscoring the importance of perceived threat, children suffering from asthma who were from low SES backgrounds were more likely to interpret ambiguous situations as threatening. However, gene expression was more strongly associated with the perception of threat than with SES itself (Chen et al., 2008). Finally, short allele carriers may be disposed to rumination, directing preferential attention toward threat-related stimuli and disengaging from such stimuli with greater difficulty (Beevers, Wells, Ellis, & McGeary, 2009; Osinsky et al., 2008). Taken together, this literature is consistent with the proposition that short allele carriers should be more hypervigilant and more reactive to early cumulative SES-associated risk than should carriers of the long or very long genotype, and so may show stronger or more prolonged physiological and psychological responses to high levels of early cumulative SES risks in childhood, leading to a greater impact of early cumulative SES risk on genome-wide methylation and depression pathway specific methylation.

Functional Impact of Changes in Methylation

Changes in methylation can exert an effect on phenotypes by changing the accessibility of the protein-coding portion of genes, or by changing the accessibility of promoter regions of genes, rendering them more or less available for genetic transcription, and so potentially changing function or developmental trajectory. Conversely, changes in methylation may change functioning of particular genes or cellular processes by facilitating or inhibiting the production of interfering proteins, which may also have potential regulatory impact. From the standpoint of understanding long-term changes in behavioral tendencies or health outcomes, patterns of methylation associated with early cumulative SES risks are of particular interest, because some alterations in methylation may remain stable over a relatively long time in humans (Eckhardt et al., 2006), making individual differences in methylation a potential biological marker of specific early environmental contributions to later outcomes (Fraga et al., 2005).

To the extent that epigenetic change results from the interaction of genotype with early cumulative SES risk,

methylation should provide a sensitive index of $G \times E$ effects, and perhaps result in more replicable patterns (cf. Kinnally et al., 2010) than can be observed using downstream behavioral phenotypes, like depression, that may depend critically on additional subsequent developmental and environmental processes. If so, examination of epigenetic change potentially may be able to tease apart subtle differences in patterns of effects (cf. Koenen et al., 2011), such as the differences in patterns predicted by vulnerability and susceptibility frameworks. An added benefit is that a focus on epigenetic change may decrease concerns about measurement and methodological confounds that sometimes render self-reported behavioral outcomes less compelling as critical tests of theory. Therefore, examination of hypothesized effects of interactions between early cumulative SES risk and 5-HTTLPR on the methylation of particular genetic pathways relevant to depression, and to developmental outcomes more broadly, may provide a useful supplement to a focus on behavioral outcomes, while also providing potential clues regarding etiology.

Vulnerability Versus Susceptibility

The observations reviewed above regarding the impact of the short allele on the response to stress suggest a *vulnerability* or *diathesis–stress* model for understanding the impact of variation at 5-HTTLPR in response to conditions of high adversity. A genetic vulnerability model of 5-HTTLPR effects posits that short allele carriers are less resilient to high levels of stress, placing them at increased risk of adverse outcomes, perhaps including enhanced epigenetic change, in the context of elevated early cumulative SES stress. From a vulnerability perspective, there would be no reason to expect differences to emerge in the absence of heightened stress. Recently, however, an alternative to the vulnerability framework has been proposed in the form of a susceptibility framework. Although related, and generating several overlapping predictions, genetic susceptibility models differ from genetic vulnerability models in one key respect: they predict differential response to low-stress, positive environments as well. A susceptibility model of the impact of variation at 5-HTTLPR on epigenetic outcomes would posit that carriers of the short allele are genetically predisposed to be more susceptible than others to a range of environment influences. Therefore, compared to others, short allele carriers would be expected to demonstrate greater propensity for epigenetic change in response to elevated early cumulative SES risk, a prediction that overlaps with those from a vulnerability model. In addition, short allele carriers would also be expected to demonstrate a significant difference in the opposite direction in response to lower stress, positive early environments, leading to a “for better or worse” pattern of outcomes (Belsky & Pluess, 2009; Boyce & Ellis, 2005). The difference between these two models is illustrated in Figure 1 and Figure 2. Both vulnerability and susceptibility perspectives predict an interaction between 5-HTTLPR and early cumulative SES, and both predict greater impact of variation in early cumulative SES risk among short

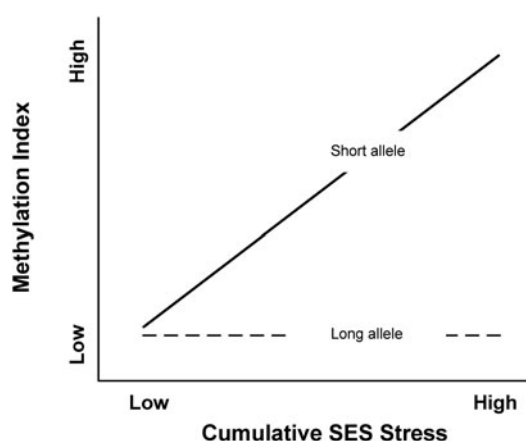


Figure 1. The prototypical vulnerability effect. No difference in outcome at low stress but divergence at high stress are shown. SES, socioeconomic status.

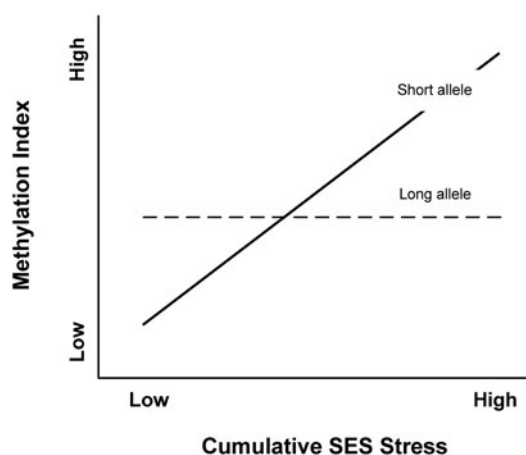


Figure 2. The prototypical susceptibility effect. Differences in outcome at low stress and divergence at high stress are shown, but in opposite directions. SES, socioeconomic status.

allele carriers. However, only the susceptibility model suggests that genotype will also be associated with significant differences in methylation at low levels of early cumulative SES risk, and that the direction of difference between short carriers and those with only long or very long alleles when exposed to low levels of early cumulative SES risk will be the reverse of that seen at high levels of early cumulative SES risk.

Quantification of Comparisons of Vulnerability and Susceptibility

Quantification of the differences between the vulnerability and the susceptibility models has been the focus of recent methodological work, resulting in suggestions that comparisons of the vulnerability and the susceptibility models should provide four key pieces of information. Such comparisons should provide significance tests for the interaction term in addition to visual inspection of the interaction plot, report re-

gions of significant difference between the simple slopes in addition to reporting the simple slopes and the significance of the simple slopes, report the proportion of the area between the regression lines uniquely attributable to susceptibility effects in order to characterize the relative importance of susceptibility effects compared to vulnerability effects, and introduce regression terms that directly examine potential nonlinearity and rule out nonlinear effects or significant interaction effects that might masquerade as crossover effects (Roissman et al., 2012).

Effect on the Depression Pathway

Prior work has identified a large set of genes associated with depression in at least one study (the Kyoto Encyclopedia of Genes and Genomes [KEGG] pathway databases; Kanehisa & Goto, 2000). In preliminary research, we examined the impact of early cumulative SES risk on CpG sites across this pathway (Beach et al., 2013). A set of 50 cytosine nucleotide–phosphate–guanine nucleotide (CpG) sites on the depression pathway were associated significantly with the main effect of early cumulative SES risk. An index reflecting degree of methylation across these 50 CpG sites fully mediated the impact of early cumulative SES risk on depressive symptoms in young adulthood (see Beach et al., 2013, for details). This main effect pathway linking early cumulative SES risk to depression provides an interesting starting point for the exploration of moderating effects of the *5-HTTLPR* on epigenetic change. It provides a main effect that is neutral with regard to the presence or direction of any interaction with genotype. In addition, it provides a direction of negative effect from early cumulative SES risk to later depressive symptoms, providing interpretive advantages. Specifically, examination of the impact of the short allele on methylation across this main effect index can be evaluated for direction and pattern of impact as well as for magnitude of impact.

An alternative approach to the comparison of the susceptibility versus the vulnerability models is examination of an interaction index created from all CpG sites on the depression pathways that demonstrate a significant association with the interaction term, thereby capturing the full conditional effect of cumulative SES risk that varies by genotype. Although a negative (i.e., depressogenic) direction of effect cannot be specified a priori, it is possible to construct an interaction index that reflects the differential effect of cumulative SES risk on methylation of CpG sites on depression-related genes for those with an short allele. This interaction index allows for an examination of the overall shape of the epigenetic interaction effect, contrasting impact for short allele carriers to that observed for those with only long or very long alleles. Because they represent independent tests of the shape of the interaction, consistent patterns between main effect and interaction effect indices suggest robustness of conclusions. Finally, because the *5-HTTLPR* may have broader implications for developmental outcomes, it is useful to conduct exploratory analyses on a genome-wide basis to test the hypothesis that

short allele carriers will show increased impact of early cumulative SES risk at the genome-wide level of analysis and that these effects may have broader developmental significance. Effects can be clarified further through characterization of the genetic pathways that are differentially changed.

Specific Hypotheses

Based on the foregoing considerations, we hypothesized that genetic variation at the *5-HTTLPR* would act as either a vulnerability or a susceptibility factor in relation to the epigenetic impact of early cumulative SES risk. In particular, it was expected that variation at the *5-HTTLPR* would interact with early cumulative SES risk to create differential methylation of the CpG sites on the depression main effect pathway, resulting in a stronger effect on the overall pathway, as well as a stronger effect of early cumulative SES risk on methylation when examined on a locus-by-locus basis. Further, we hypothesized that graphical representation would indicate either relatively equal effects of variation at *5-HTTLPR* at low versus high cumulative SES, indicating increased susceptibility among short allele carriers, or significant impact of variation at *5-HTTLPR* only at high cumulative SES, indicating increased vulnerability. In addition, we hypothesized that variation at the *5-HTTLPR* would interact with early cumulative SES risk to create differential DNA methylation of the depression interaction effect index and would replicate the shape of the main effect pathway, confirming either vulnerability or susceptibility patterns. Specifically, we anticipated converging support for either increased susceptibility or increased vulnerability among short allele carriers. We also expected to replicate findings for increased magnitude of effect on methylation among short allele carriers relative to others. Finally, we hypothesized that interaction of early cumulative SES risk with *5-HTTLPR* on methylation across the genome would reflect differential change in pathways having neurodevelopmental significance and that short allele carriers would again show enhanced impact of the environmental variable on epigenetic reprogramming, this time at the genome-wide level.

Method

Participants

African American primary caregivers in rural Georgia from each of $N = 388$ families participated in data collection and provided parental assessments of the economic circumstances of the family. Youth in the target age range were selected and provided genetic and epigenetic assessments. Target youth mean age was 11.7 years at the first assessment, 15.6 years at the time of genotyping base on Oragene collection, and 19.2 years at the time of epigenetic assessment based on a blood draw. The 388 families participating in the current study resided in nine rural counties in Georgia in which poverty rates are among the highest in the nation and unemploy-

ment rates are above the national average (Proctor & Dalaker, 2003). At the first assessment, primary caregivers in the sample worked an average of 39.9 hr per week, and 42.3% lived below federal poverty standards. The proportion below the poverty standard was 56.3% at the time of epigenetic assessment. The increase in poverty is attributable to worsening economic conditions in the region. Of the youth in the sample, 55% were female. When targets were 11 years old, 78.8% of the caregivers had completed high school or had earned a GED, and median monthly family income was \$1,710. Median monthly income was \$1,648 at the age 19 data collection. The families, on average, could be characterized as working poor.

The analytic sample was drawn from a larger, ongoing data collection involving 667 families who had been recruited from lists of fifth-grade students provided by local schools (see Brody et al., 2004, for a full description). Due to budgetary constraints, we selected subsamples for biological assessment from the set of all youth available at the age 18 and 19 data collections ($N = 561$, a retention rate of 84%). We selected a random subsample of 500 to be assessed on a range of biological measures other than blood draws, and a subsample of these ($N = 399$) were selected to participate in blood draws to allow methylation analyses. The 388 youth included in the current analyses participated in the blood draw and had complete data on all measures.

The full count (and proportion) of each genotype at the 5-HTTLPR is as follows: for short, short (S, S), 21 (0.054); for S, long (S, L), 127 (0.327); for S, very long (VL), 4 (0.01); for L, L, 220 (0.567); for L, VL, 14 (0.036); and for VL, VL, 2 (0.005). The distribution of short versus long alleles did not deviate from Hardy-Weinberg equilibrium ($\chi^2 = 0.22$, *ns*), suggesting no problem with differential allele drop out. Consistent with prior research (Brody et al., 2011; Hariri et al., 2005), genotyping results were used to form two groups of participants: those with only long allele or very long alleles (i.e., L, L; L, VL; and VL, VL; coded as 0, $n = 236$, 60.8%) and those with either one or two copies of the short allele (i.e., S, S; S, L; S, VL; coded as 1, $n = 152$, 39.2%).

Procedure

All data were collected in participants' residences using a standardized protocol that lasted approximately 2 hr at each wave of data collection. Two African American field researchers worked separately with the primary caregiver and the target youth. Interviews were conducted privately, with no other family members present or able to overhear the conversation. Primary caregivers consented to their own and the youths' participation in the study, and the youths assented to their own participation and consented when they participated as adults.

Measures

Preadolescent early cumulative SES risk. Three waves of data collected from primary caregivers when the target youths

were 11, 12, and 13 years of age were used to establish level of early cumulative SES risk across six indicators, with each indicator scored dichotomously (0 if absent, 1 if present; see Evans, 2003; Kim & Brody, 2005; Rutter, 1993; Sameroff, 1989; Werner & Smith, 1982; Wilson, 1987). Early cumulative SES risk was defined as the average number of risk factors across the three assessments, yielding an index with a theoretical range of 0 to 6 ($M = 2.33$, $SD = 1.35$). The six risk indicators were (a) family poverty, defined as being below the poverty level, taking into account both family income and number of family members; (b) primary caregiver noncompletion of high school or an equivalent; (c) primary caregiver unemployment; (d) single-parent family structure; (e) family receipt of Temporary Assistance for Needy Families; and (f) income rated by the primary caregiver as not adequate to meet all needs.

Genotyping. Most participants' DNA ($N = 343$) was obtained at age 16 using Oragene DNA kits (Genotek; Calgary, Alberta, Canada). Participants rinsed their mouths with tap water, then deposited 4 ml of saliva in the Oragene sample vial. The vial was immediately sealed, inverted to allow mixing with stabilizing agents, and shipped via courier to a central laboratory in Iowa City, where samples were prepared according to the manufacturer's specifications. Genotype at the 5-HTTLPR was determined for each sample as described previously (Bradley, Dodelzon, Sandhu, & Philibert, 2005) using the primers F-GGCGTTGCCGCTCTGAATGC and R-GAGGGACTGAGCTGGACAACCAC, standard *Taq* polymerase and buffer, standard dNTPs with the addition of 100 μ M 7-deaza GTP, and 10% DMSO. The resulting polymerase chain reaction products were electrophoresed on a 6% nondenaturing polyacrylamide gel, and products were visualized using silver staining. Two individuals who were blind to the study hypotheses and other information about the participants called the genotypes. For the 45 participants who were not successfully genotyped at age 16 using the Oragene method, genotypes were assayed in the same manner except using blood drawn at age 19.

Characterization of methylation. Certified phlebotomists went to each participant's home to draw a blood sample comprising four tubes of blood for a total of 30 ml of whole blood. After the blood was drawn into serum separator tubes, it was shipped the same day to the Psychiatric Genetics Lab at the University of Iowa for preparation. After receipt of the blood at the University of Iowa, each tube was inspected to ensure anticoagulation, and aliquots of blood were diluted 1:1 with phosphate buffered saline pH 8.0. Mononuclear cell pellets were then separated from the remainder of the diluted blood specimen by centrifugation with ficoll at 400 $g \times 30$ min and the mononuclear cell layer was removed from the tube using a transfer pipette, resuspended in a phosphate buffered saline solution, and briefly centrifuged again. The resulting cell pellet was resuspended in a 10% DMSO/RPMI

solution and frozen at -80°C until use. Typical DNA yield for each pellet was between 10 and 15 μg of DNA.

Genome-wide DNA methylation was assessed using the Illumina (San Diego, CA) HumanMethylation450 Beadchip under a subcontract to the University of Minnesota Genome Center (Minneapolis, MN) using coded DNA specimens. Two replicate samples of an internal control DNA were included in each plate to aid in assessment of batch and chip variation and to ensure correct handling of specimens. The average correlation coefficient between the replicate samples was greater than 0.99. DNA from the subjects was randomly assigned to slides holding DNA for 12 subjects, with groups of eight slides representing the samples from a single 96-well plate that had been bisulfite converted in a single batch. The resulting data were inspected for complete bisulfite conversion, and beta values (i.e., average methylation) for each CpG residue was determined using the GenomeStudio V2009.2; Methylation module Version 1.5.5, Version 3.2 (Illumina, San Diego). The resulting data were cleaned to remove those beta values whose detection p values (an index of the likelihood that the observed sequence represents random noise) were greater than .05, using a PERL-based algorithm. Data were inspected for outliers or confounds by plate and chip variables. With respect to this sample, more than 99.76% of the 485,577 probes yielded statistically reliable data. Beta values were exported into Microsoft Excel, and initial analyses were conducted in R. To remove noninformative CpG sites as well as CpG sites of reduced potential predictive validity (Plume, Beach, Brody, & Philibert, 2012), all CpG sites with average methylation less than 5% (47,275 CpG sites) or greater than 95% (11,632 CpG sites) were removed from the data set. This resulted in a data set of 426,670 informative CpG sites, with methylation values between 5% and 95%, from which data for our genome-wide analyses and our analyses of depression pathways was drawn.

Depression pathway. To create the depression-related gene pathway score, we identified all genes associated with depression using the KEGG databases (Kanehisa & Goto, 2000). Because individual genes are of small effect and may be conditioned by other genes and risk factors, we followed the approach suggested in the KEGG database and included all genes appearing in genome-wide association studies and other investigations reporting a positive association, regardless of replication status. The depression pathway was identified as having 222 genes, 208 of which were assessed by the Illumina array. The association of methylation with cumulative SES-related risk was examined for all CpG sites on the 208 depression-related genes, allowing characterization of significance level and direction of association. Using the R-2.13.1 software package, with log10-transformed methylation values as the dependent measure and no control variables, all CpG sites that were significantly associated with cumulative SES-related risk at the $p < .01$ level were included in the depression-SES pathway index, allowing identification of CpG sites that were elevated above the median in the direction of

the association, and assignment of 0 versus 1 for each CpG site identified. For each CpG site significantly associated with SES-related risk, if the association was positive, all those scoring above the median had pathway scores incremented by 1; if the association was negative, all those scoring below the median had pathway scores incremented by 1. This yielded for each person the number of CpG sites that had been methylated in the direction associated with greater cumulative SES-related risk. Summing across CpG sites resulted in an index score with a potential range of 0 to 50; the observed range was 1 to 47 ($M = 24.619$, $SD = 10.79$).

To create an index of association with the interaction term, all CpG sites that were significantly associated ($p < .01$) with the interaction of cumulative SES risk and presence of an short allele were included in the depression-interaction pathway ($N = 300$). This index is a count of CpG sites associated with cumulative SES risk. When cumulative SES risk was positively associated with methylation among short allele carriers, individuals above the mean of the distribution had index scores incremented by 1 and otherwise 0. Conversely, for CpG sites that were negatively associated with cumulative stress among short allele carriers, index scores were incremented by 1 when the methylation value for the individual was below the mean and 0 otherwise. Therefore, the theoretical range of the interaction pathway score was 0–300 and the observed range was 11–289. Higher index scores were obtained by individuals who most closely conformed to the high cumulative SES risk, short allele, methylation prototype.

Results

Comparisons of short and long alleles

As an initial step in the analyses, we examined whether there were any significant differences between short allele carriers and other youth with regard to economic risks and related variables. As can be seen in Table 1, there were no significant differences between the two groups, although there was a trend for less parental unemployment among the short allele carriers.

Effects on the main effect depression pathway

We next examined the impact of the interaction between presence of an short allele and presence of early cumulative SES risk on methylation of CpG sites on genes in the depression main effect pathway. In preliminary work (Beach et al., 2013), we found that higher values on the index were associated with greater earlier cumulative SES risk and with greater young adult depressive symptoms.

The impact of 5-HTTLPR on the association of early cumulative SES risk with level of the main effect methylation index is reported in Table 2. On Step 1, we regressed the depression main effect index score for each participant on early cumulative SES risk and 5-HTTLPR genotype (short allele carrier = 1 vs. other = 0), as well as age and gender. We

Table 1. Student *t* tests and descriptive statistics comparing components of the cumulative risk index for short allele carriers versus those with no short allele

Variables	5-HTTLPR				<i>t</i>
	S Allele (<i>n</i> = 152)		No S Allele (<i>n</i> = 236)		
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	
Age at the first assessment	11.63	0.36	11.67	0.35	−1.21
Hours work per week	39.97	11.16	39.89	10.89	0.064
Percent female	0.50	0.50	0.58	0.49	−1.55
Family incomes	2071.52	1570.09	2039.71	1399.43	0.20
Poverty 150% below limit	0.71	0.46	0.67	0.47	0.88
Parent unemployment	0.18	0.38	0.25	0.43	−1.63
Single-parent family	0.59	0.49	0.59	0.49	0.036
TANF receipt	0.07	0.26	0.08	0.27	−0.29
Parent education < high school	0.53	0.50	0.51	0.50	0.35
Inadequate income for needs	0.37	0.48	0.33	0.47	0.82

Note: All components are not significantly different based on a Student *t* test ($p < .05$). 5-HTTLPR, serotonin transporter linked polymorphic region; S, short; TANF, Temporary Assistance for Needy Families.

then examined the moderating effect of variation at the 5-HTTLPR by entering the product of cumulative SES risk, centered, and 5-HTTLPR genotype on a second step. We used bootstrapping methods to determine standard errors, allowing

simultaneous estimates of all effects and avoiding the assumption of a standard normal distribution when calculating *p* values. We used 1,000 resamples of the data, and used bias-corrected and accelerated bootstrap confidence intervals

Table 2. Bootstrapping regression models with robust standard errors explicating the impact of cumulative socioeconomic status risk, 5-HTTLPR, and their interaction on methylation of the main-effect depression pathway, controlling for age and gender

	Model 1	Model 2	Model 3
	Unstand. <i>b</i> (95% CI)	Unstand. <i>b</i> (95% CI)	Unstand. <i>b</i> (95% CI)
Main effect			
Cumulative SES risk (ages 10–13)	3.487** (2.433, 4.540)	2.364** (0.926, 3.802)	2.429** (0.950, 3.907)
Cumulative SES risk squared			−0.190 (−1.366, 0.986)
5-HTTLPR (1 = SS, SL)	−0.265 (−2.321, 1.792)	−0.287 (−2.315, 1.740)	0.260 (−2.455, 2.976)
Two-way interaction			
Cumulative SES Risk × 5-HTTLPR		2.901** (0.855, 4.948)	2.799** (0.736, 4.862)
Cumulative SES Risk Squared × 5-HTTLPR			−0.559 (−2.429, 1.311)
Control variables			
Age	0.930 (−0.754, 2.614)	0.911 (−0.743, 2.565)	0.914 (−0.0736, 2.546)
Gender	−1.508 (−3.618, 0.601)	−1.364 (−3.437, 0.709)	1.350 (−3.427, 0.726)
Constant	25.396** (23.842, 26.950)	25.329** (23.762, 26.896)	25.514** (23.618, 27.411)
<i>R</i> ²	0.118	0.135	0.138

Note: Bootstrap = 1,000 replications; average risk index (Waves 1–3) is standardized by *z* transformation, and age is measured by mean centering; 5-HTTLPR, serotonin transporter linked polymorphic region gene; CI, confidence interval; SES, socioeconomic status; SS, short-short; SL, short-long. *N* = 385.

** $p \leq .01$ (two-tailed test).

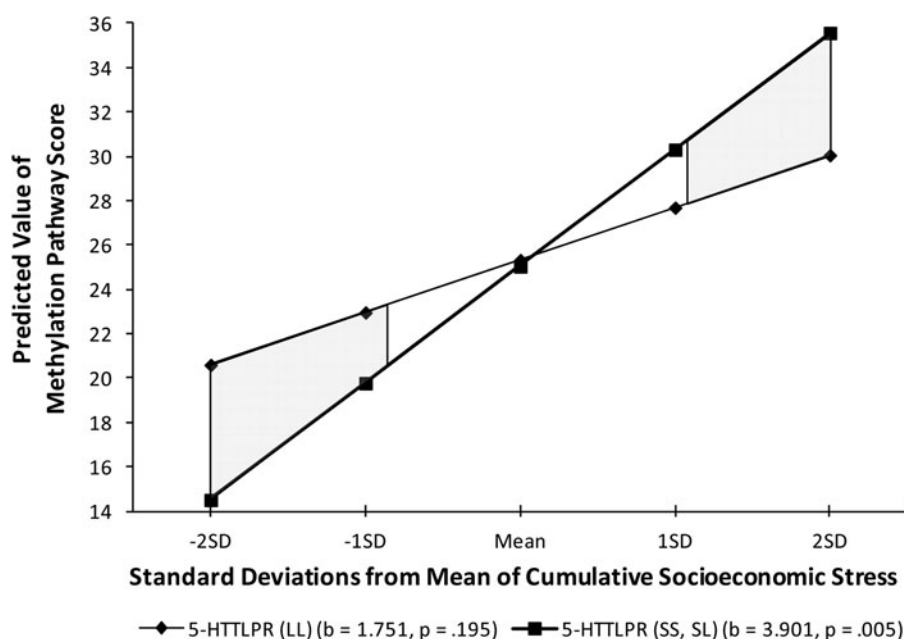


Figure 3. The moderating role of variation in the serotonin transporter linked polymorphic region gene (*5-HTTLPR*) on the association of early cumulative socioeconomic status risk with the main effect methylation index for the depression pathway (50 cytosine nucleotide–phosphate–guanine nucleotide sites). Higher scores reflect methylation in the direction mediating the effect of SES on future depressive symptoms. Shaded regions reflect significant differences between the regression lines. LL, long–long; SS, short–short; SL, short–long.

(95%) to adjust for any bias in the sampling distribution. As can be seen in Table 2, the main effect for early cumulative risk was significant on Step 1 ($b = 3.487$, $\beta = 0.323$, $p = .0001$), and the interaction with the *5-HTTLPR* genotype was significant on Step 2 ($b = 2.901$, $\beta = 0.167$, $p < .005$).

To explicate the significant interaction and identify regions of significant difference, we first examined simple slopes. As can be seen in Figure 3, the simple slopes conformed to expectations of greater impact of cumulative SES risk among short allele carriers. The significant interaction effect is the result of a steeper and significant slope for the association of early cumulative SES risk with the depression main effect index among those carrying the short allele ($b = 3.901$, $p = .005$), but a less steep and nonsignificant slope among those with only long or very long alleles ($b = 1.751$, ns). In addition, there is a crossover effect of the sort predicted by the susceptibility model. That is, short allele carriers experiencing low levels of cumulative SES risk demonstrated less methylation in the depressogenic direction than did those carrying only long and very long alleles. Conversely, short allele carriers experiencing higher levels of early cumulative SES risk demonstrated greater methylation in the depressogenic direction than did those carrying only long and very long alleles. Although the simple slopes are in the same direction for short allele carriers and those carrying only long and very long alleles, there is a significantly greater effect of early cumulative SES risk on the overall main effect index among short allele carriers.

In keeping with evolving methodological guidelines, we further compared the vulnerability versus the susceptibility

models by highlighting regions of significant difference between the two simple regression lines. These are shown as shaded areas between the simple regression lines in Figure 3, and they indicate substantial and symmetrical regions of significant difference at both ends of the early cumulative SES risk continuum. We also computed the proportion of interaction index to compare the proportion of significantly different effects uniquely attributable to effects at both ends of the early cumulative SES risk continuum. The resulting ratio of smaller region to total shaded region was 0.45, close to the maximum potential value of 0.5, suggesting a relatively robust finding in favor of the susceptibility model. Finally, as can be seen in Table 2 (Step 3), we repeated the regression analyses introducing nonlinear effects and found these effects to be nonsignificant.¹ Specifically, the quadratic term formed

1. It is possible that nonlinearity could be introduced if a more extreme range of values were examined. It seems likely that nonlinearity will always be critically dependent on the range of environments examined as well as the scoring of the dependent variable. In the current sample, early cumulative SES risk was pronounced and resulted in a roughly equal number of youth who were profoundly and not profoundly affected by SES stress. It is also worth noting that interactions reflecting vulnerability (but not susceptibility) effects can be eliminated or substantially reduced by transforming the dependent variable. Accordingly, interaction tests using transformed data may provide an alternative way of controlling for cryptic nonlinearity. We also ran the analyses for the main effect index including sex as a predictor and allowing it to interact with all other variables. In that analysis, there was a significant three-way interaction of Sex \times Cumulative SES Risk \times *5-HTT*, which indicated that effects were in the same direction for males and females but stronger for males. However, this pattern was not replicated in

by early cumulative SES risk squared and the interaction of the quadratic term with 5-HTTLPR genotype were both nonsignificant. Despite this, the previously significant interaction effect of genotype with early cumulative SES risk remained significant after introduction of the nonlinear terms, suggesting that it is not attributable to underlying curvilinear effects.

We next examined the impact of 5-HTTLPR genotype on the association of early cumulative SES risk and methylation on a locus-by-locus basis across the main effect index. For 42 out of 50 CpG sites, the absolute value of the correlation of cumulative risk with methylation was greater for short allele carriers than for those carrying only long or very long alleles. The interaction effect reflected relatively greater impact of cumulative SES risk on DNA methylation among short allele carriers at the aggregate level as well as at the level of individual CpG sites.

A second stage in our examination of whether the effect of early cumulative SES risk on depression pathway methylation was moderated by 5-HTTLPR was to test whether the pattern reported above could be replicated using all CpG sites on depression-associated genes for which methylation level was significantly associated with the interaction of early cumulative SES risk and 5-HTTLPR. A series of regression analyses was used to examine the 4,661 CpG sites on depression-related genes, introducing the interaction of genotype and early cumulative risk on a second step, after first entering genotype, early cumulative SES risk, age, and gender. The 300 CpG sites for which a nominally significant ($p < .01$) interaction effect emerged, controlling for main effects, were used to form the interaction index examined next.

The interaction index comprised CpG sites ($n = 300$) scored as 1 for CpG sites that were positively associated with cumulative stress for short allele carriers and for which the individual was above the mean of the distribution, and otherwise 0. Conversely, CpG sites that were negatively associated with cumulative stress for short allele carriers were scored 1 when the methylation value for the individual was below the mean and scored 0 otherwise. Higher index scores were obtained by individuals who most closely conformed to the high-risk short allele methylation prototype.

To examine the shape of the interaction effect across depression-related genes, we regressed the depression interaction index score for each participant on early cumulative SES risk and the 5-HTTLPR genotype, as well as the control variables of age and gender, on Step 1. We then examined the moderating effect of variation at the 5-HTTLPR by entering the interaction of cumulative risk and genotype on the second step of the regression. As before, we used bootstrapping methods to allow simultaneous estimates of all effects and

to avoid reliance on assumptions of a standard normal distribution when calculating p values. We used 1,000 resamples of the data, and bias-corrected and accelerated bootstrap confidence intervals (95%) to adjust for any bias in the sampling distribution. As can be seen in Table 3, the main effect for early cumulative SES risk was significant on Step 1 ($b = 7.479$, $\beta = 0.103$, $p = .046$), and the interaction with the presence of a short allele at the 5-HTTLPR genotype was also significant on Step 2 ($b = 39.003$, $\beta = 0.333$, $p < .0001$).

Figure 4 explicates the interaction effect. As can be seen, short allele carriers showed, on average, a greater response to early cumulative SES risk. The significant interaction effect is the result of a steeper and significant slope for the association of early cumulative SES risk with the depression interaction index among those carrying the short allele ($b = 31.39$, $p = .0001$), but a less steep and nonsignificant slope among those with only long or very long alleles ($b = 7.62$, ns). In addition, there was a crossover pattern (i.e., the pattern indicative of susceptibility effects). To further clarify the comparison of the vulnerability versus the susceptibility models, we highlighted regions of significance, showing substantial regions of significant effects at both ends of the early cumulative risk continuum. We also computed the proportion of interaction index. The resulting value of 0.49 suggests a robust finding in favor of susceptibility. Finally, we repeated the analyses including potential nonlinear effects as before, and again found them to be nonsignificant. There was no significant effect of a quadratic term for early cumulative SES risk, nor did the quadratic term interact significantly with the 5-HTTLPR genotype.

It should be noted that, unlike the analyses with the main effect index, the interaction index is scored in a manner that ensures a more positive association between the index score and early cumulative SES risk for short allele carriers. However, it should also be noted that this need not translate into an advantage in favor of the susceptibility model relative to the vulnerability model, nor does it necessarily produce greater absolute correlations for short allele carriers. Examination of the relative strength of associations at the level of individual CpG sites produced an additional useful consistency check. Across the 300 CpG sites on the interaction pathway, 277 CpG sites demonstrated a significantly greater absolute correlation for short allele carriers than for youth with only long or very long alleles. The results replicate the pattern observed in the main effect pathway analysis, suggesting that genotype at the 5-HTTLPR is consequential for shape of epigenetic impact of early cumulative SES risk on the depression pathway, that the presence of an short allele confers greater impact of early cumulative SES risk on epigenetic change, and that the susceptibility model better captures the pattern of moderation than does the vulnerability model, with effects present across a majority of CpG sites on the pathway.

To complete our examination of 5-HTTLPR effects on depression pathway methylation, we also examined the effect of carrying an short allele on the relative impact of early cumulative SES risk on methylation on each of the 4,661 CpG sites

the subsequent analysis using the interaction effect index; for that analysis, there were no significant interactions with sex. Nor was the stronger effect for males observed in the genome-wide examination. In that analysis, the moderating effect of the short allele was more pronounced for females than for males, although once again it was in the same direction.

Table 3. Bootstrapping regression models with robust standard error explicating the impact of cumulative socioeconomic status risk, 5-HTTLPR, and their interaction on methylation of the interaction-effect depression pathway, controlling for age and gender

	Model 1	Model 2	Model 3
	Unstand. <i>b</i> (95% CI)	Unstand. <i>b</i> (95% CI)	Unstand. <i>b</i> (95% CI)
Main effect			
Cumulative SES risk (ages 10–13)	7.479* (0.135, 14.822)	−7.615 (−16.849, 1.618)	−9.004† (−18.619, 0.611)
Cumulative SES risk squared			3.975 (−4.028, 11.978)
5-HTTLPR (1 = SS, SL)	−0.612 (−15.747, 14.524)	−0.919 (−15.456, 13.618)	6.239 (−13.041, 25.520)
Two-way interaction			
Cumulative SES risk × 5-HTTLPR		39.003** (25.074, 52.931)	40.221** (26.044, 54.397)
Cumulative SES Risk Squared × 5-HTTLPR			−7.218 (−21.282, 6.846)
Control variables			
Age	2.214 (−9.495, 13.924)	1.959 (−9.257, 13.174)	2.374 (−8.952, 13.701)
Gender	11.256 (−3.739, 26.251)	13.193† (−1.108, 27.493)	13.729† (−0.609, 28.068)
Constant	136.785** (125.964, 147.607)	135.880** (125.278, 146.482)	131.658** (118.442, 144.873)
<i>R</i> ²	0.017	0.084	0.088

Note: Bootstrap = 1000 replications; average risk index (Waves 1–3) is standardized by *z* transformation, and age is measured by mean centering; 5-HTTLPR, serotonin transporter linked polymorphic region gene; CI, confidence interval; SES, socioeconomic status; SS, short-short; SL, short-long. *N* = 385.

†*p* ≤ .10. **p* ≤ .05. ***p* ≤ .01 (two-tailed test).

on the depression pathway (i.e., not just those that were significantly associated with main or interaction effects). Specifically, we examined whether the pattern of differences in absolute correlation values across the entire pathway was consistent with a greater impact of early cumulative risk among carriers of the short allele than for those who did not have an short allele. Of 4,661 CpG sites examined, 3,528 demonstrated greater absolute correlations between early cumulative risk and methylation for short allele carriers, representing a significant deviation from the null expectation that there would be equal numbers of CpG sites with greater and lesser impact for each genotype.

Finally, to investigate whether there was a pattern of increased impact among short allele carriers on a genome-wide basis and whether the effect of carrying the short allele was consistent with the expectation of enhanced impact on developmentally important processes among short allele carriers, we conducted genome-wide analyses to identify all CpG sites associated with the interaction effect at the *p* < .01 level. Using a series of regressions to examine each loci across the filtered data set, and using the same analytic approach as for the previous analyses, with main effects entered prior to the centered interaction effect, we identified 25,601 CpG sites out of the set of 426,670 total filtered CpG sites that were significantly (nominally) associated at the *p* < .01

level with the interaction of early cumulative SES risk and genotype controlling main effects, age, and sex.

Among the set of 25,601 significantly associated CpG sites, we examined whether the magnitude of association was greater for short allele carriers than for those carrying only long or very long alleles. For each CpG site, we examined the difference between short allele carriers and those carrying only long or very long alleles in the absolute value of the correlation of cumulative risk with methylation. In 23,864 CpG sites, the short allele carriers demonstrated greater absolute impact of cumulative risk on degree of methylation than did their long or very long allele counterparts, deviating sharply from expectations under the null hypothesis. Conversely, only 1,737 CpG sites showed the reverse pattern, with short allele carriers demonstrating a smaller association of early cumulative SES risk with degree of methylation. The strong shift toward greater impact among short allele carriers genome-wide can be seen in the histogram in Figure 5. The height of the bars in the histogram indicates the number of CpG sites for which the difference in magnitude of correlations is within each given range. As can be seen, the modal difference is between 0.1 and 0.2, and the median value is 0.149.

The sixfold greater number of significant CpG sites associated with the interaction effect genome-wide relative to

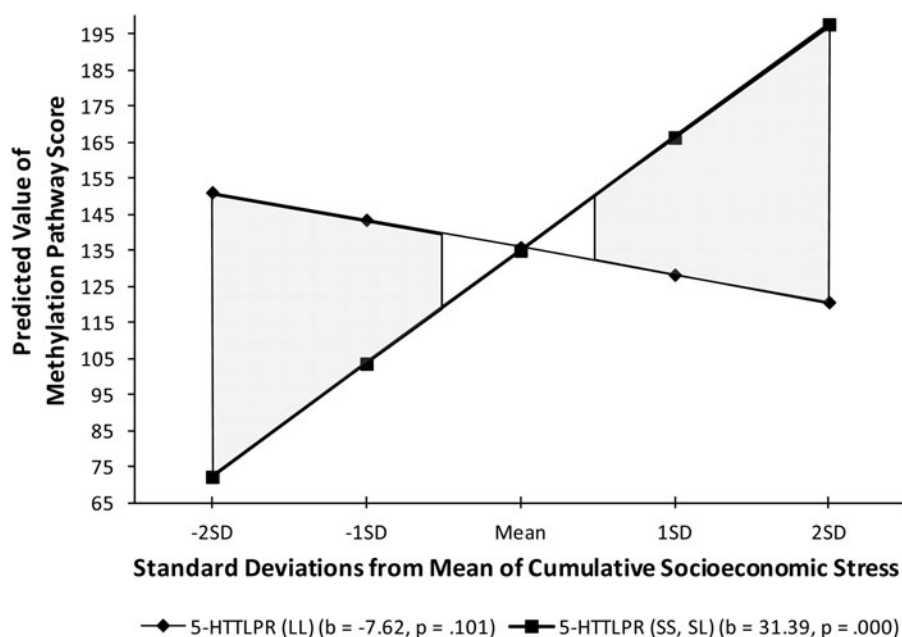


Figure 4. The moderating role of variation in the serotonin transporter linked polymorphic region gene (*5-HTTLPR*) on the association of early cumulative socioeconomic status risk with the interaction-related methylation index for the depression pathway (300 cytosine nucleotide–phosphate–guanine nucleotide sites). Higher scores reflect methylation in the direction of a positive association for short allele carriers. Shaded regions reflect significant differences between the regression lines. LL, long–long; SS, short–short; SL, short–long.

chance suggested the potential value of further characterization of the effect within a gene pathway analytic framework. To examine significantly enriched pathways using controls for multiple comparisons, we examined the significantly associated CpG sites in GoMiner™ using default settings (i.e., 0.05 settings for reports and all gene ontology as the root category setting) and using the 25,601 CpG sites identified in the genomewide analyses as the “changed” gene set (Zeeberg et al., 2003).

The top 30 pathways are reported in Table 4, with nominal and false discovery rate corrected values reported. As can be seen, there was a systematic patterning in the methylation data reflecting the influence of the interaction between genotype and early cumulative SES risk on a number of key developmental epigenetic pathways.

Among the top 10 pathways showing differential impact as a function of the *5-HTTLPR* were nervous system development, cellular developmental process, neuron projection, de-

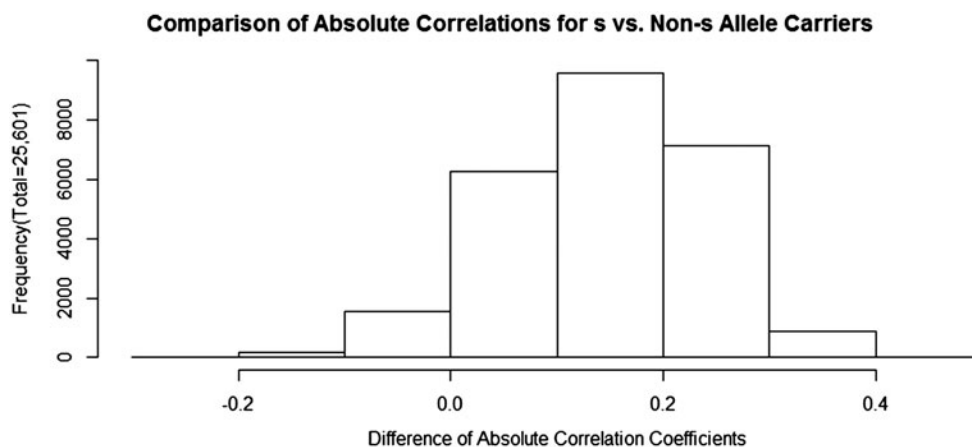


Figure 5. A histogram of the differences in correlation magnitude for short allele carriers versus others across the 25,601 cytosine nucleotide–phosphate–guanine nucleotide sites demonstrating a significant interaction of variation in the serotonin transporter linked polymorphic region gene with cumulative socioeconomic status risk. The median value is 0.149, indicating greater magnitude for short allele carriers. s, short; non-s, not short.

Table 4. The top 30 most differentially regulated gene ontology pathways for interaction effects of early cumulative stress with the serotonin transporter

GO Category	Category Name	Genes			
		Total	Changed	Log ₁₀ <i>p</i>	FDR
GO:0030154	Cell differentiation	2825	1532	−21.2044	0
GO:0007399	Nervous system development	1905	1070	−20.5701	0
GO:0048869	Cellular developmental process	2894	1562	−20.5269	0
GO:0043005	Neuron projection	603	384	−18.3279	0
GO:0032502	Developmental process	4935	2533	−18.2893	0
GO:0007275	Multicellular organismal development	4513	2329	−17.9512	0
GO:0009653	Anatomical structure morphogenesis	2302	1253	−17.7078	0
GO:0048731	System development	3782	1975	−17.5666	0
GO:0023052	Signaling	4769	2443	−16.8903	0
GO:0048856	Anatomical structure development	4238	2186	−16.4639	0
GO:0048468	Cell development	1586	888	−16.4326	0
GO:0022008	Neurogenesis	1226	703	−16.0295	0
GO:0048699	Generation of neurons	1153	665	−15.9134	0
GO:0042995	Cell projection	1124	650	−15.8964	0
GO:0030182	Neuron differentiation	1082	626	−15.3683	0
GO:0044459	Plasma membrane part	2338	1252	−14.6631	0
GO:0045202	Synapse	494	312	−14.27	0
GO:0023060	Signal transmission	3620	1871	−14.1181	0
GO:0023046	Signaling process	3624	1872	−14.0017	0
GO:0005515	Protein binding	8633	4224	−13.9081	0
GO:0032989	Cellular component morphogenesis	965	559	−13.8783	0
GO:0000902	Cell morphogenesis	882	515	−13.6359	0
GO:0007154	Cell communication	2062	1106	−13.115	0
GO:0051239	Regulation of multicellular organismal process	1615	884	−13.0582	0
GO:0000904	Cell morphogenesis involved in differentiation	747	442	−12.9906	0
GO:0048666	Neuron development	857	499	−12.9368	0
GO:0009887	Organ morphogenesis	995	568	−12.4929	0
GO:0030030	Cell projection organization	931	533	−12.0308	0
GO:0044463	Cell projection part	540	329	−11.9901	0
GO:0031175	Neuron projection development	736	431	−11.7343	0

Note: FDR, false discovery rate. There were 518 differentially regulated gene ontology pathways based on FDR < 0.01.

developmental process, multicellular organismal development, anatomical structure morphogenesis, and system development. Therefore, the GoMiner analyses suggest both coherent patterning of methylation effects associated with the *5-HTTLPR* by early cumulative SES risk interaction effect and a concentrated impact on development.

Discussion

The goal of the current investigation was to examine several interrelated questions regarding the potential impact of genetic variation at *5-HTTLPR* on long-term vulnerability to depression and neurodevelopmental outcomes in response to early cumulative SES risk. Because the long-term effects of early cumulative SES risk are well known and the assessment of such risk is based on relatively objective indicators, this investigation provides a useful context for exploration of claims regarding the magnitude, shape, and generality of the impact of genetic variability at the *5-HTTLPR* on epigenetic change. We focused on epigenetic change in depression-related genes as an outcome of interest because this contributes directly to

the continuing discussion of the nature, consistency, and mechanisms of *5-HTTLPR* effects. The findings were consistent with the expectation of enhanced impact of early cumulative SES risk among carriers of the short allele. The effect was examined in several ways, and consistent patterns were observed. Whether examining the main effect pathway (i.e., the 50 CpG sites identified previously on the basis of significant main effects and association with young adult depression) or examining the interaction pathway (i.e., the 300 CpG sites that were significantly associated with the interaction effect across the depression pathway), the full set of all 4,661 CpG sites sampled from depression-related genes, or all CpG sites across the genome associated with differential impact of early cumulative SES risk, there was evidence of increased impact of early cumulative SES risk on methylation among short allele carriers relative to those with only long or very long alleles. Therefore, the finding that the short allele of the *5-HTTLPR* is associated with increased impact of early cumulative SES risk on methylation appears robust.

We also used the methylation data to construct indices to compare the susceptibility and the vulnerability models at

the level of epigenetic change among depression-associated genes. Consistent support was found for the susceptibility model relative to the vulnerability model. For both the main effect index and the interaction effect index, we found evidence of crossover effects.² The effect of early cumulative risk on methylation was significant among short allele carriers but not among those who did not carry a short allele. In addition, for both indices, regions of significant difference were identified at both ends of the early cumulative SES risk continuum, the proportion of the interaction effect uniquely associated with susceptibility was substantial, and there was no evidence of confounding by nonlinear effects. Therefore, for the depression pathway indices, each of the four types of evidence currently recommended for comparison of vulnerability and susceptibility models supported the susceptibility perspective.

Using GoMiner, we conducted gene pathway analyses using the information from the 25,601 probes that were nominally differentially methylated at the $p < .01$ level owing to the interaction of the 5-HTTLPR and early cumulative SES risk. A theme of the most significantly differentially methylated pathways was that neuronal and developmental pathways were differentially influenced by early cumulative SES risk, depending on the presence of the short allele. As shown by the pattern of differences in absolute correlations, these differences once again reflected the stronger impact of early cumulative SES risk among short allele carriers. Overall, 518 gene pathways survived false discovery rate correction for the interactive effect of 5-HTT and cumulative SES risk on genomewide methylation, suggesting that the impact of the short allele on response to early cumulative risk may extend well beyond its effects on depression-related genes. However, caution is required in the interpretation of the GoMiner results, because the approach used in these analyses focuses on number of genes enriched relative to the genome as a whole, discarding potentially important information about degree of change and number or pattern of CpG sites affected. The approach also assumes the independence of affected genes and pathways, an assumption that is likely violated in most cases for analyses of patterns of methylation. Thus, the GoMiner results may best be viewed as providing an indication of the relative likelihood of differentially affected gene pathways rather than as providing an absolute p value for individual pathways.

The current study was designed to detect small to medium effect sizes (e.g., $r^2 = .05$). For an effect of this size, the current sample ($N = 385$) provides power greater than 0.90. In the current analyses, both the main effect of cumulative SES risk ($\beta = 0.219$) and its interaction with 5-HTTLPR ($\beta = 0.167$) had medium to large effects in the prediction of

the main effect index reported in Table 2. In addition, the effect size associated with the interaction of cumulative SES risk and with 5-HTTLPR ($\beta = 0.333$) was large for the analysis reported in Table 3 (i.e., the interaction index). Power calculations using Monte Carlo simulation procedures (Muthen & Muthen, 2002) indicated that we had power greater than 0.90 to detect these effects. Similarly, in the context of separately testing vulnerability and susceptibility components of the model in Figures 3 and 4, power was estimated to be greater than 0.80. Conversely, consistent with the broader literature on genetic main effects, the main effect of variation at 5-HTTLPR is quite small ($\beta = -0.013$ and $\beta = -0.006$), yielding a power estimate of 0.079 to detect the effect in the context of Table 1 and 0.066 in Table 2 for a sample of this size. Therefore, the current sample would be underpowered to detect the main effect of genotype on indices of methylation, but it appears to be adequately powered to detect the interaction effects of cumulative SES risk and genotype. At the same time, the current sample is substantially underpowered for genome-wide examination of effects on individual CpG sites owing to the need to correct for multiple comparisons, and this is one reason it is not attempted in the current manuscript. Recent reviews have questioned the role of 5-HTTLPR in the prediction of depression and other forms of psychopathology (Risch et al., 2009). In particular, it has been noted that there have been a number of nonreplications of studies using complex phenotypes, like depression, as the outcome variable and complex stressors, such as life stress or early adversity, as the environmental variable. In the current report, we focus on a simple and relatively objective childhood stressor, early cumulative SES risk, and a simple biological dependent variable, DNA methylation. Because the main effect of early cumulative SES risk on a range of outcomes is not in dispute, we are able to focus on whether variation in the 5-HTTLPR acts as a vulnerability or susceptibility factor in the context of a well-known childhood stressor. In addition, a focus on DNA methylation has several advantages in the context of testing the susceptibility hypothesis. Because it does not involve self-report, it is immune to concerns regarding reactivity and self-report biases. Therefore, the current report's focus on epigenetic change provides an alternative to self-reported symptoms and behavior in tests of the effect of the short allele at the 5-HTTLPR and whether it acts as a susceptibility or vulnerability factor. In addition, because methylation changes may be functional, observed differences also have the potential to illuminate potential etiological pathways. Finally, because epigenetic change may influence activity in a number of genetic pathways, it provides a context for exploration of broader patterns of epigenetic influence.

The pattern of results is clear in supporting the role of 5-HTTLPR as a susceptibility factor in response to variation in exposure to early cumulative SES risk. In a relatively impoverished sample with high representation of working poor, we find that presence of the 5-HTTLPR short allele is associated with significantly different and greater impact of early cumulative SES risk on gene methylation. It is worth

2. It is of potential theoretical interest to compare models of absolute versus relative deprivation to examine whether susceptibility effects for cumulative SES risk depend on having a substantial proportion of the population under the poverty level, as was the case in the current sample, or whether susceptibility effects emerge as well owing to variation in circumstances within populations with higher average SES and fewer individuals below the poverty level.

noting that the sampling strategy conforms to calls for strategies that increase the potential power to detect $G \times E$ effects by focusing on samples with optimal distribution of exposure variables (Caspi et al., 2010). However, it should be noted as well that the CpG sites examined are a small subsample of all potential methylation sites, creating potential for future tests with more comprehensive coverage to lead to different results or to identify subsets of CpG sites that respond differently.

Several limitations of the current investigation are important to note. For example, these findings are based on a relatively small sample size with limited statistical power, particularly with regard to determination of genome-wide levels of significance. We observed differential association between cumulative SES risk and methylation for carriers of short and long alleles at multiple CpG sites, but none of the associations at individual CpG sites reached the level of statistical significance required to confirm the association as genome-wide significant. Consequently, our conclusions reflect inferences for sets of CpG sites rather than individual CpG sites. A focus on sets of CpG sites across functionally related genes, as illustrated in the current report, provides a potentially useful framework for future research by behavioral researchers working with modest sample sizes. In addition, because DNA methylation patterns exert different effects on gene transcription depending on where they are located, it cannot be assumed that all significant methylation effects had similar associations with gene transcription of the affected gene. Therefore, the connection between observed changes in pathway methylation and gene activity remains to be confirmed for most of the genes examined (see Plume et al., 2012).

There are also several challenges to the identification of mechanisms of change using the current data. There is the potential problem of tissue specificity of epigenetic control (e.g., Davies et al., 2012). In particular, we cannot assume that methylation patterns observed in lymphocytes are similar in all respects to the patterns likely to be observed in other tissues of particular interest, such as specific types of brain tissue. Clearly, some methylation patterns are likely to be tissue specific because of the important role of DNA methylation in the specialization of tissue type. However, Rollins, Martin, Morgan, and Vawter (2010) have shown that peripheral measures of gene expression predict central expression for genes

expressed similarly in each cell type. Shumay, Logan, Volkow, and Fowler (2012) found that methylation of monoamine oxidase A in white blood cells predicted brain monoamine oxidase A levels, suggesting that peripheral measures of methylation can predict brain endophenotype. Likewise, Davies et al. (2012) found sufficient correspondence in intraindividual variation across brain and blood to indicate the utility of peripheral tissues in epidemiological studies. Therefore, for CpG sites on sets of genes that are similarly expressed, patterns of methylation may be more similar than different across different tissue types. Nonetheless, the degree to which, and the circumstances under which, methylation patterns observed using lymphocytes reflects methylation in particular regions of interest in the brain will require additional direct investigation.

These limitations notwithstanding, the current results suggest that methylation is an excellent vehicle for studying potential long-term developmental effects of early stressors. It appears possible that some long-term effects of early stressors may be mediated by their impact on changes in methylation patterns across the genome that result in shifts in developmental processes or that directly influence later behavioral and health outcomes. The current study supports continued exploration of epigenetic change as a mechanism accounting for $G \times E$ effects on development and long-term effects. Better understanding the role of epigenetic change in functional genetic pathways will require additional investigation at multiple levels of analysis with a particular focus on the time course of methylation change, the extent to which particular patterns are fixed or malleable, and investigation of the time frame over which change may be reversible. It will be important to identify appropriate ages for detection of particular outcomes. For example, whereas detection of associations between methylation and some outcomes, like depression, might be readily observed in young adulthood, other outcomes may not be readily observed until later in life. Likewise, it will be important to better specify sets of genes or gene pathways most directly related to behavioral phenotypes and outcomes of interest. In brief, although epigenetic change appears to be a promising mechanism for future exploration, it will require better incorporation into a broad developmental psychopathology framework to reach its full potential.

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