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Prevention of early substance use mediates, and variation at *SLC6A4* moderates, SAAF intervention effects on *OXTR* methylation

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Abstract

The Strong African American Family (SAAF) program has been shown to have a variety of short and long-term benefits for participating youth and families. However, biological mechanisms potentially influencing long-term effects on resilience in young adulthood have not been examined. In the current investigation, we examine the effects of SAAF on methylation of the *OXTR* gene in young adulthood, focusing on a regulatory region previously identified to be both responsive to stress and implicated in resilience. Using the subsample of participants from the original study for whom methylation data was available ($N = 388$), we replicated the previously reported $G \times E$ effect on prevention of early substance use and then examined whether there would also be a moderated effect on *OXTR* methylation in early adulthood, with “s” allele carriers, but not “LL” participants, showing a significant indirect effect of SAAF on *OXTR* methylation. Results suggest that for susceptible youth (i.e., “s” allele carriers), preventive intervention may “get under the skin,” in a manner potentially beneficial for long-term outcomes. Implications for examination of *OXTR* methylation in future prevention research is discussed.

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Keywords

OXTR; *SLC6A4*; prevention; substance use; family

Preventing early onset of substance use is important because of its potential to reduce long-term problematic outcomes in adolescence and adulthood (Brook & Newcomb, 1995; Choi, Gilpin, Farkas & Pierce, 2001) and risky behavior later in life. The Strong African American Families Program (SAAF) was designed to prevent the initiation of a cluster of early risk behaviors including alcohol use, marijuana use, and sexual activity, and has been found to be successful in doing so (Brody, Murry, Kogan, Gerrard, Gibbons, Molgaard, ... Wills, 2006; see also MacPhee, Lunkenheimer & Riggs 2015 for a review of similar programs that replicate these effects in other groups). Once effects are established, better understanding mechanisms of change increases opportunities to strengthen and refine successful preventive interventions. In this regard, both psychosocial and biological pathways are relevant to understanding SAAF effects.

Considerable work has been done to explicate psychosocial pathways mediating SAAF effects on early and adolescent outcomes, with findings suggesting that key changes include SAAF induced reductions in exposure to deviant peers and risky peer situations and increased protective interactions with parents (see Brody, Beach, Philibert, Chen, & Murry, 2009). However, biological markers and mechanisms associated with SAAF's effects, and particularly markers that may forecast later positive health behaviors and adult psychosocial resilience are not yet well documented. Accordingly, using a sample randomly assigned to SAAF or control conditions, we examined the program's impact on *OXTR* methylation 9 years later. *OXTR* methylation was selected as a potential biomarker for the activity of the oxytocin system, a system implicated in a range of affiliative, resilience, and long-term health outcomes (Kumsta, Hummel, Chen, & Heinrichs, 2013).

Oxytocin

Oxytocin is a peripheral hormone and central neuromodulator that influences a number of social and affective responses relevant to social support and resilience. The Oxytocin system has been studied in numerous ways. Intranasally administered oxytocin has been found to increase optimism, trust, generosity, and empathy and to reduce anxiety (Baumgartner, Heinrichs, Vonlanthen, Fischbacher, & Fehr, 2008; Kosfeld, Heinrichs, Zak, Fischbacher, Fehr, 2005; MacDonald & MacDonald, 2010) whereas naturally occurring variation in oxytocin is associated with greater attachment and has anxiolytic effects in the context of partner support (Tops, Van Peer, Korf, Wijers, Tucker, 2007). Because only one oxytocin receptor has been identified (i.e., *OXTR*), the level of activity of this gene is believed to be of particular importance in regulating the activity of the oxytocin system.

Highlighting its potential role in facilitating resilience, a higher level of oxytocin increases the likelihood that threatening situations will be perceived as challenges rather than as stressors (Olf, et al., 2013). Likewise, the importance of *OXTR* in substance use and other long-term outcomes of interest is suggested by the high density of *OXTR* in the cortex, hippocampus, limbic system, and amygdala among other regions thought to be involved in

regulating mood and addictive behavior (Sarnyai, 2011). Further, in animal models, variation in oxytocin influences development of tolerance to opioids and alcohol as well as presence of withdrawal symptoms (Kovacs, et al., 1998), and may have potential to inoculate against future vulnerability to substance use (McGregor & Brown, 2012). At the same time, the oxytocin system has been shown to regulate cognitive and emotional responses, reduce activation of the amygdala, and augment connectivity between the amygdala and brain structures supporting affective control (Gamer, Zurowski, and Buchel, 2010; Puglia et al., 2015), all potential longer-term outcomes of interest to prevention researchers.

Suggesting bidirectional connections between the oxytocin and the serotonin systems, experimental administration of oxytocin produces shifts throughout the 5-HT system by down regulating the amygdala (Mottollese, et al., 2014) and there is strong input from the serotonin system into the oxytocin system in controlled laboratory settings (Galfi et al., 2005). Accordingly, to the extent that SAAF produces changes in the long-term regulation of the oxytocin system, it has substantial potential to confer long-term benefits in a range of domains of interest to prevention science, and given the connection with the serotonin system, such effects may be particularly beneficial for carriers of the “s” allele of the 5-HTTLPR.

OXTR Methylation

One potential mechanism by which SAAF could influence or regulate the oxytocin system, possibly with long-term implications, is through epigenetic effects at *OXTR*. Methylation of specific CpG sites (i.e., regions of DNA in which cytosine occurs next to guanine separated by only one phosphate bond), can influence access to key regulatory elements controlling the rate of gene transcription, and so influence gene expression and larger systemic effects. Because greater methylation of the first exon is particularly likely to predict lower gene expression (e.g., Brenet et al., 2011), characterizing individual differences in methylation of *OXTR* in the region of the first exon may be particularly informative, and so provides a useful starting point for examination of potential biomarkers of SAAF’s impact on the oxytonergic system. In keeping with this general expectation, Kusui, Kimura, Ogita, Nakamura, Matsumara, Koyama, ... Murata, (2001) found that methylation of the CpG island surrounding the first exon of *OXTR* has a substantial effect on gene transcription, and that the strongest methylation effects were attributable to methylation of a sub-region around the end of the first exon and extending into the first intron.

Having a specific regional focus is important in examination of methylation of CpG sites across *OXTR* because methylation patterns for this gene have been shown to be multi-dimensional, with several factors accounting for variation in methylation across the gene (e.g., Cecil, et al., 2015; Kim et al, 2014; Simons et al., in press). Based on the prior data, we hypothesized that we would identify a set of CpGs that loaded together on a single factor representing physically contiguous loci located on or around the end of the first exon of *OXTR*, suggesting strong potential for regulatory impact. Prior research has identified a set of three loci on the first exon that are both stress responsive and associated with social/affiliative schemas in adults (see Simons, et al., in press), making them an ideal candidate set.

SAAF Treatment Effects are Moderated by 5-HTTLPR

The role of genetic polymorphisms in response to intervention has been highlighted and explored in prior research (IJzendoorn, et al., 2012). One genetic polymorphism that has received attention is the “s” allele in the promoter region of the serotonin transporter gene (*SLC6A4*), also referred to as the 5-HTT-linked polymorphic region (i.e., the 5-HTTLPR), a key regulator of serotonergic neurotransmission. This polymorphism results in two common variants, a long (*l*) and a short (*s*) allele, as well as some less common variants (e.g., the very long (*vl*) allele), and some variations with less pronounced effects on gene expression (i.e., the A/G substitution, see Philibert et al. 2008). The “s” variant is associated with lower availability of 5-HTT and reduced efficiency of 5-HTT reuptake.

There is prior evidence linking genetic variation in the serotonin transporter to differential susceptibility in response to the SAAF prevention program (Brody, et al., 2009), and Beach et al. (2014) found that the “s” allele also was associated with differential impact of SES related risk factors on methylation, suggesting that biological embedding may be influenced by presence of the “s” allele. Accordingly, we anticipated a similar pattern of genetic moderation for the effect of SAAF on methylation at *OXTR*. Further supporting the expectation of moderated effects of SAAF on methylation of *OXTR*, low serotonin is implicated in disruptions of social behavior among primates and humans (Raleigh, McGuire, Brammer & Yuwiler, 1984), suggesting that “s” allele carriers may be more vulnerable to social disruption and so more likely to experience benefits in response to SAAF, increasing effects on *OXTR* methylation.

Moderation of Substance Use Effects by 5-HTTLPR

Adolescents with a short allele of the 5-HTTLPR are at increased risk for substance use initiation in the context of early life stress (Kaufman, et al., 2007), with the effect stronger among adolescents experiencing lower levels of supportive parenting (see Brody et al., 2009). Among those participating in SAAF, the interaction of participation in the program with presence of the “s” allele resulted in earlier onset of substance use among “s” allele carriers who were in the control group relative to “s” allele carriers receiving SAAF or non-carriers in either group. More recent evidence suggests that “s” allele carriers may continue to be at elevated risk for onset of additional risky behavior if they initiate substance use. In particular, among youth initiating early substance use, 5-HTTLPR was found to interact with early substance use initiation such that carriers of the “s” allele who were early initiators were also more likely to initiate risky sexual behavior two years later (Kogan et al., 2010). Accordingly, carrying the “s” allele may be involved in further amplification of problems associated with early substance use initiation, potentially amplifying effects on affiliation related biomarkers, leading to the pattern of multiple moderation illustrated in Figure 1.

Context for the Current Investigation

Economic disadvantage is widespread in the Southeastern coastal plain, leading many African American youth living in the Southeast to experience a range of stressful, recurrent, and uncontrollable difficulties at developmentally sensitive stages. This context may also

influence outcomes and potentiate negative effects of carrying the “s” allele for *SLC6A4* (Beach et al., 2014). Accordingly, experience of SES related adversity during early development is controlled in all analyses. Similarly, a range of circumstances may give rise to variation in the proportion of different cell types in the blood samples used to assess methylation (Beach et al., 2015), and it is important to control such variability in analyses linking environments to change in methylation of particular genetic regions. Accordingly, controls for cell-type variability are also included in all analyses. Finally, there is the potential for cryptic population stratification to better account for effects than does a specific genotype, and so we also include a control for ancestry informative genetic markers in the current analyses. Likewise, controls for age and sex are included in all analyses.

Specific Hypotheses

We examine three main hypotheses. First, we examine the effect of SAAF on early initiation of substance use, predicting that it will be moderated by the presence of the “s” allele in the 5-HTTLPR, replicating prior analyses with the full sample. Second, we examine the effect of early initiation of substance use on methylation of *OXTR* in young adulthood, predicting that its impact also will be moderated by variation at the 5-HTTLPR, extending prior research on mechanisms by which genotype may influence long-term trajectories and amplify the impact of early contexts on young adult outcomes. Third, we examine the indirect effect of participation in the SAAF program on methylation of *OXTR* in young adulthood through prevention of early substance use. We predict that this effect will be significant only among carriers of the “s” allele at the 5-HTTLPR, and not among non-carriers, leading to a significant mediated moderation effect. In all cases we control for gender, variation in youth exposure to SES-risk, genetic ancestry, and individual differences in cell-type composition of white blood cell pellets where appropriate.

Methods

Participants

Data were collected as part of the evaluation of the SAAF family-based preventive intervention study and subsequent follow-up evaluations. Participants resided in nine rural counties in Georgia. In these counties, families live in small towns and communities in which poverty rates are among the highest in the U.S. and unemployment rates are above the national average (Proctor & Dalaker, 2003). In the current study, the analytic sample was drawn from a larger, ongoing data collection (see Brody et al., 2006, for a full description). Due to budgetary constraints, we selected a subsample for biological assessment ($N = 399$), and these participants provided blood draws allowing us to conduct methylation analyses. The sample eligible for the current study included the 388 families randomly assigned to SAAF or control who participated in the blood draw, completed data on all measures, and were successfully genotyped at 5-HTTLPR. Target youth mean age was 11.7 years at the first assessment, 13.2 at the assessment of substance use initiation, 15.6 years at the time of genotyping base on Oragene collection, and 20.5 years at the time of epigenetic assessment based on a blood draw. At the first assessment, 42.06% lived below federal poverty

standards. The proportion below the poverty standard was 56% at the time of epigenetic assessment. Of the youth in the final sample, 55% were female.

Procedure

All data were collected in participants' residences. Two African American field researchers were used for each family to allow separate and simultaneous data collection from the primary caregiver and the target youth. In addition, interviews were conducted 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.

Intervention Implementation and Fidelity—The SAAF prevention program consisted of seven consecutive meetings held at community facilities, with separate parent and youth skill-building curricula and a family curriculum. Concurrent and family sessions each lasted 1 hour; and so parents and youths received 14 hours of prevention training. Details are described by Brody et al. (2006).

Measures

Early Substance use initiation—Participants reported whether they had ever drunk beer, wine, wine coolers, whiskey, gin, or other liquor (alcohol use); or used marijuana (Brody et al., 2009). These items were scored dichotomously as 1 for yes (ever used alcohol, or used marijuana) and 0 for no. The two items were summed to form a substance use initiation index that could range from 0 to 2 at each assessment. The mean substance use initiation index score was .13 ($SD = .34$) at pretest (age 11) and .34 ($SD = .54$) at wave 3 (age 13).

Genotyping—Most participants' DNA ($N = 343$) was obtained at age 16 using Oragene™ DNA kits (Genetek, Calgary, AB, Canada), 45 participants were genotyped using blood drawn at age 19. Genotype at the *5-HTTLPR* was determined for each sample as described previously (Bradley, Dodelzon, Sandhu, & Philibert, 2005). Consistent with prior research (Brody et al., 2009), we treated genetic variation at the *5-HTTLPR* as a dichotomous variable, 1 for those carrying at least one copy of the short allele and 0 for those homozygous for the long allele. Among the 388 respondents, 5.4% were homozygous for the short allele (*ss*), 33.8% were heterozygous (*sl*), and 60.8% were homozygous for the long allele (*ll*). Using the Hardy-Weinberg equilibrium test, the observed distribution of *5-HTTLPR* did not differ significantly from that predicted on the basis of simple Mendelian inheritance.

Ancestry informative genetic markers—Because population genetic admixture may confound G×E findings (Halder et al., 2009), we used the Structure program, version 2.3.4 (Falush, Stephens, & Pritchard, 2007) with a panel of 24 ancestry informative markers to estimate an ancestry proportion for each participant. Three ancestral populations (African, European, and Other) were found to be relevant to characterizing ancestry, and so racial admixture variables (Ancestry proportion 1, Ancestry proportion 2) were used as covariates in the analyses. Average level of European Ancestry was 28.9%

Methylation Assessment—When participants were 20.5 years old, a certified phlebotomist drew four tubes of blood (30 ml) from each participant and shipped it the same day to a laboratory for preparation. Typical DNA yield for each pellet was between 10 and 15 mg of DNA. The Illumina (San Diego, CA) 450K HumanMethylation Beadchip was used to assess Genome-wide DNA methylation. The average correlation coefficient between replicate samples was 0.99 and data were cleaned using the Illumina Genome Studio Methylation Module, Version 3.2. Quantile normalization (e.g. Pidsley et al., 2013) was used to correct distributional problems inherent in array data using the watermelon (2013) R package (Team, 2012) and the “dasen” function recommended by Pidsley et al. (2013). Following quantile normalization, no technical variability (chip/batch effects) remained in array data.

OXTR Methylation—Using annotation provided in the Illumina 450K HumanMethylation Beadchip, 12 CpG sites were found to be within the *OXTR* associated CpG island. Consistent with prior studies (Kim et al, 2014; Simons et al., in press), the results of a principal component analysis suggested four orthogonal factors with eigenvalues greater than 1. Tabled results of the PCA are available in the supplemental material (Table S1). Consistent with prior research we identified a set of three CpGs (factor 4; cg09353063, cg17285225, and cg23391006) that cohered as a factor, were physically contiguous, and were located on the first exon of *OXTR*, suggesting strong potential for regulatory impact. The location of these loci is shown in the supplemental material (Figure S1), and they are identical to the set found previously in a different sample of African American adults by Simons et al. (in press). Accordingly, we standardized and then averaged the beta scores for each of the three loci to form our index of *OXTR* methylation.

Control variables—Covariates included gender and cumulative socioeconomic risk (SES-related risk). SES-related risk was defined as the sum of six socioeconomic risk indicators: family poverty as assessed using U.S. government criteria (an income-to-needs ratio < 1.5), primary caregiver non-completion of high school or an equivalent, primary caregiver unemployment, single-parent family structure, family receipt of “Temporary Assistance for Needy Families”, and income rated by the primary caregiver as less than adequate to meet all needs. In addition, we also controlled for cell type where appropriate. Ficoll purified peripheral mononuclear cell DNA pellets of the sort used in the current investigation are comprised of several different cell types (CD4, CD8, CD14, CD19, CD56) (Reinius, et al., 2012), leading to potentially spurious associations if individual differences in predictors and outcomes are also associated with individual differences in mixture of cell types. To account for individual differences in cell types, we utilized the regression calibration approach utilized by Dogan et al., (2014) based upon the work of Houseman and colleagues (2012). This approach yielded four factors informative with regard to the relative distribution of (CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells and CD56+ Natural Killer cells). These four factors were included as controls in our analyses focused on *OXTR* methylation.

RESULTS

Descriptive and association analysis

The means, standard deviations, and zero order correlations among the study variables are presented in Table 1. Using independent *t*-tests, there were no mean differences between carriers of the *s* allele and *ll* allele homozygotes on any of the study variables at the baseline assessment. As expected, participation in SAAF was associated with changes in early substance use and *OXTR* methylation was associated with early substance use for carriers of the 5-HTTLPR “*s*” allele, but were not significantly correlated for *ll* carriers (simple correlations within genotypes provided in the supplemental materials table S2). There was no significant correlation of “Percent European Ancestry” with substance use initiation.

Gene-environment correlations

Because gene-environment correlations (*r*GE) can confound assessment of gene-environment interactions (Caspi et al., 2010), we examined bivariate associations. There was no direct association of genotype with SAAF or early initiation of substance use at baseline, suggesting that random assignment was successful in eliminating potential confounding due to *r*GE.

H1: Amplification of the effect of SAAF on early initiation of substance use

Regression analyses were used in Models 1a and 1b in Table 2 to examine prediction of early initiation of substance use (at wave 3). We first checked for potential multicollinearity among variables. VIF scores ranged between 1.00 for early initiation of substance use SES-related risk and 1.07 for admixture proportion, and all measures of tolerance were above .90, suggesting that multicollinearity was not present. To examine the shape of the interaction effect of SAAF with genotype on early initiation of substance use, we first regressed the early initiation of substance use score at age 13 for each participant on SAAF participation (0 vs. 1), 5-HTTLPR genotype (*s* allele carriers = 1; others = 0), wave 1 substance use, and the control variables of gender and SES risk exposure. We then examined the moderating effect of 5-HTTLPR by entering the interaction of SAAF and genotype in the second step of the regression. As can be seen in Table 2, model 1a, the main effects for SAAF and genotype were nonsignificant. However, in model 1b, the interaction of SAAF participation with the presence of the “*s*” allele was significant. Figure 2A depicts the interaction effect. As can be seen, *s* allele carriers showed, on average, a greater response to SAAF. There were significant differences in early substance use initiation between those with differing genotype only for those in the control group. Those in the treatment condition did not show significant differences in substance use initiation related to genotype, replicating prior analyses with the full sample (Brody, et al., 2009).

H2: Amplification of early substance use on methylation of *OXTR*

Using methylation of *OXTR* as the outcome, we again used regression analyses to examine main and interaction effects, beginning by checking for multicollinearity. The VIF ranged from 1.01 to 1.15, and all measures of tolerance were above .85, indicating no multicollinearity. As can be seen in Model 2a of Table 2, neither the main effect of early

initiation of substance use (age 13), nor genotype was significant. In Model 2b we added the multiplicative interaction term for substance use by genotype. As hypothesized, the interaction was significant even after controlling for ancestry and cell-type variation. Exclusion of outliers greater than three standard deviations from the mean on *OXTR* methylation ($n = 8$) left the pattern of significant results unchanged (See supplemental Table S3) and so all participants were retained.

We plotted *OXTR* methylation as a function of substance use initiation at age 13 ranging from 2 *SD* below to 2 *SD* above the sample mean. As can be seen in Figure 2B, the significant interaction effect resulted from a significant slope for the association of early initiation of substance use with methylation of *OXTR* among those carrying the “s” allele ($b = .20, p = .01$), but a negatively-signed and non-significant slope among those with only the long allele ($b = -.05, p = .51$). The grey area in the figure shows that for those with an “s” allele, earlier initiation was associated with greater methylation at *OXTR*.

H3: Conditional indirect effect of SAAF on *OXTR*

Given the presence of significant moderation by the *5-HTTLPR* genotype at both stages of the model, we examined whether the mediational component of the model presented in figure 1 would function significantly differently for *s*-allele carriers compared to *ll*-allele homozygotes, resulting in moderated mediation. To examine whether the observed differences for “s” allele carriers and others were significant, we used the moderated-mediation model outlined by Preacher et al. (2007; Model 5, p. 194). Using a bootstrapping technique with 3,000 replications, the results indicated that there was a significant difference between the indirect effect of SAAF on *OXTR* methylation for “LL” homozygotes (indirect effect = $-.003$, 95% CI $[-.023, .004]$ NS), compared to the effect for respondents who carried at least one copy of the “s” *5-HTTLPR* allele (indirect effect = $-.033$, 95% CI $[-.109, -.001]$). Accordingly, the pattern reflects moderated mediation, with significant mediation of SAAF effects on *OXTR* methylation only among “s” allele carriers.

We used a Monte Carlo simulation to evaluate statistical power to detect the key hypothesized interaction effects, finding that statistical power for the interaction effects were greater than 90% (Substance use initiation $\times$ *5-HTTLPR* = .947; SAAF $\times$ *5-HTTLPR* = .914).

DISCUSSION

Methylation of *OXTR* is associated with participation in SAAF through its effect on substance use initiation. This both illustrates an avenue by which family-based prevention programs may become biologically embedded, and highlights methylation of *OXTR* as a potentially useful biomarker of the impact of such programs. Methylation of the oxytocin receptor (*OXTR*), a gene known to be involved in social skills and cognition relating to empathy, trust, and optimism (MacDonald & MacDonald, 2010), was a natural choice as a biomarker of SAAF induced change. In keeping with prior research on genetic susceptibility for behavioral effects of SAAF, the current study investigated whether carriers of the “s” allele of the *5-HTTLPR* would also show a different pattern of epigenetic change at *OXTR* in response to SAAF. Given the strong connection between serotonergic and oxytonergic

systems (Galfi, et al., 2005), we posited that methylation of *OXTR* would be greater for those initiating early substance use to the extent they also carried the “s” allele, but would be less for those protected against early substance use initiation by participation in SAAF, or who were LL homozygotes. Thus, we expected that the pattern would highlight a significant indirect effect of SAAF on *OXTR* methylation among “s” allele carriers only, leading to a significantly moderated pattern of mediation. Differential methylation for those facing different environmental circumstance follows a general pattern in which behavioral traits are modified by experience to better fit with environmental demands (Landecker and Panofsky, 2013; Meloni, 2014; Simons & Klopach, 2015), a pattern that may be more pronounced for some environmental effects among carriers of the “s” allele of the 5-HTTLPR (Beach et al., 2014).

We focused on variation in methylation of three CpG sites in the first exon of *OXTR* because prior research suggested that this portion of the epigenetic signature for *OXTR* was associated with stress as well as with behavioral outcomes (Simons et al., in press), and because these elements cohered as a single factor. Of the four factors capturing significant variance in methylation across *OXTR*, only one was located on the first exon and it replicated exactly the factor previously shown to be responsive to stress and associated with behavioral outcomes (Simons et al., in press). Using this factor as our index of *OXTR* methylation, our regression analyses supported the hypothesized model. SAAF differentially predicted early substance use, and early substance use differentially predicted greater methylation of *OXTR*. Indirect effects of SAAF on methylation of *OXTR* were significant only for “s” allele carriers. This resulted in a significantly greater indirect effect of SAAF on *OXTR* methylation among carriers of the “s” allele than among those with the “ll” genotype, i.e. a pattern of moderated mediation.

The current results extend previous research on the genetic moderation of prevention effects, extending differential effects to young adulthood as well as implicating biological factors that may confer resilience later in adulthood. In addition, this is the first study to examine the process by which family-based prevention programming may become biologically embedded in pathways related to attachment. Although interpretation of the likely effect of peripheral measures of *OXTR* methylation is complex for several reasons, requiring several caveats and cautions, the current results suggest that methylation biomarkers can provide a useful window on the development of attachment related biology, and that methylation of *OXTR* may capture this effect. Because methylation was measured in a peripheral tissue, however, the current results should be interpreted with caution as they may not reflect patterns of methylation in *OXTR* that would be observed in brain tissue. Likewise, because *OXTR* methylation is likely correlated with other methylation changes across the genome, it will be important to examine changes at *OXTR* in the context of these broader patterns in future research. Further, some researchers have found a slightly different factor structure when examining *OXTR* methylation, or found associations at birth or early childhood focusing on other genomic regions (e.g., exon 2, Cecil, et al., 2015; promoter, Dadds et al., 2014). Similarly, the treatment of variation at 5-HTTLPR may have been overly simplified in the current study given the potential impact of other genetic elements that can introduce variability (e.g., the A to G substitution at rs25531 for “Long” allele carriers), leading to a large number of potential allelic variants for *SLC6A4* and adding potential noise to

examination of effects attributable to the “s” allele at 5-HTTLPR (Nakamura, et al., 2000). Future research with much larger samples will be required to tease out the likely nuanced effects attributable to these genetic variants and a focus on alternative genomic regions.

These findings have several implications for prevention science. First, they suggest that family based prevention programs like SAAF may have links to beneficial changes in the biological underpinnings of attachment, indicating that beneficial effects of some prevention programs may become biologically embedded and exert effects that will not be apparent until later in life. Supporting this view, supplemental analyses indicated that methylation of *OXTR* predicted subsequent change in risky behavior (See supplemental table S4) even after controlling for SAAF, substance use, and other control variables, encouraging further investigation of *OXTR* methylation as a factor in future resilience. Second, the results suggest that lower *OXTR* methylation may depend on successful delay of substance use initiation, particularly among those who are more vulnerable. Future research will be necessary to replicate this effect and to examine the time course of the changes in methylation to see if they may be useful as rapid indicators of program success. To the extent that other biomarkers of resilience can be identified, it will also be useful to investigate the role of SAAF in calibration of other biological systems, particularly those related to self-regulation and resilience. Likewise, it will be useful to further examine the extent to which *OXTR* methylation is associated with enhanced modulation of response to stressors and better outcomes in the context of stress across adulthood, with potentially important implications for understanding the longer-term consequences of prevention for health, health behavior, and future relationship quality.

The current results also suggest a cautionary note with regard to examination of biological embedding: i.e., that such effects may be complex. As is illustrated by the current results, individual difference variables associated with differential susceptibility (Belsky & Pluess, 2009), may render the impact of particular interventions or environments on methylation more readily apparent for some than for others. Given the potential complexity of completely characterizing differential susceptibility to preventive intervention (see Beach et al., in press), it may be helpful to utilize biomarkers to better capture effects and more fully explicate the range of positive outcomes from family-based early prevention programs. If replicated, *OXTR* methylation may prove useful for identification of long-term benefits of family-based prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Theoretical model linking SAAF to *OXTR* methylation via effect on substance use initiation. The model indicates that presence of the *5-HTTLPR* *s*-allele will be associated with greater effects at each stage, leading to a moderated mediation of SAAF effects on *OXTR* methylation.

Figure 2a

Figure 2b

Figure 2.

Figure 2a. Predicted value of substance use initiation at age 13 for control and prevention group by genotype at *5-HTTLPR*.

Figure 2b: The effect of substance use initiation at age 13 on *OXTR* methylation moderated by *5-HTTLPR* (s allele carriers vs. noncarriers)

Table 1

Correlations, Means, and Standard Deviations among Study Variables (N = 388)

	1	2	3	4	5	6	7	8	9	10	11
1. SAAF condition	—										
2. <i>OXTR</i> methylation	-.012	—									
3. <i>5-HTTLPR</i> (1 = <i>ss/sl</i>)	-.055	.036	—								
4. substance use initiation (W1)	.027	.062	.022	—							
5. substance use initiation (W3)	-.003	.029	.016	.544**	—						
6. Males	-.053	.245**	.079	-.009	-.030	—					
7. SES-related risk (W1-W3)	.041	-.087	-.004	-.012	-.007	-.031	—				
8. Factor 1 cell type	-.106*	.146**	.003	-.096 [†]	-.033	-.162**	-.080	—			
9. Factor 2 cell type	-.137**	.480**	-.044	.024	-.024	.222**	-.105*	-.003	—		
10. Factor 3 cell type	.090 [†]	.023	-.004	.043	.083	-.145**	.027	.002	.016	—	
11. Factor 4 cell type	-.061	.010	.041	-.026	.049	-.092 [†]	-.042	-.009	.001	.006	—
Mean	.606	-.005	.392	.129	.338	.451	6.863	.160	.002	.059	.031
<i>SD</i>	.489	.637	.489	.335	.540	.498	4.006	31.452	10.363	6.568	4.999

***p* .01;**p* .05;[†]*p* .10 (two-tailed tests).

Factors 1 to 4 are the four principle components reflecting cell-type variation in the current data.

Table 2

Regression models depicting moderating effects of variation at the *5-HTTLPR* on substance use initiation (model 1B) and methylation at OXTR (model 2B) controlling for the population admixture

	Substance use initiation (Age 13)				OXTR methylation (age 20)			
	Model 1a		Model 1b		Model 2a		Model 2b	
	<i>b</i>	β	<i>b</i>	β	<i>b</i>	β	<i>b</i>	β
SAAF condition	-.015 (.048)	-.013	.083 (.062)	.075	.106 $\dagger$ (.058)	.081	.113 $\dagger$ (.058)	.087
Substance use initiation (Age 13)					.056 (.051)	.047	-.045 (.067)	-.038
<i>5-HTTLPR</i> (1 = <i>ss/sl</i>)	.012 (.048)	.011	.156 * (.075)	.141	.056 (.058)	.043	-.025 (.067)	-.020
SAAF $\times$ <i>5-HTTLPR</i>			-.242 * (.097)	-.187				
Substance use initiation (Age 13) $\times$ <i>5-HTTLPR</i>							.243 * (.104)	.147
Males	-.026 (.047)	-.024	-.029 (.047)	-.027	.232 ** (.059)	.182	.232 ** (.059)	.182
SES-related risk (Ages 11–13)	.000 (.006)	-.001	.000 (.006)	.003	-.003 (.007)	-.021	-.003 (.007)	-.021
Substance use initiation (Age 11)	.875 ** (.069)	.544	.887 ** (.069)	.551				
Factor 1 cell type					.004 ** (.001)	.186	.004 ** (.001)	.184
Factor 2 cell type					.028 ** (.003)	.452	.027 ** (.003)	.445
Factor 3 cell type					.003 (.004)	.031	.003 (.004)	.031
Factor 4 cell type					.004 (.006)	.028	.003 (.006)	.026
Ancestry proportion 1	-.042 (.096)	-.020	-.039 (.096)	-.018	-.032 (.115)	-.012	-.039 (.114)	-.015
Ancestry proportion 2	-.081 (.098)	-.037	-.092 (.097)	-.042	.023 (.116)	.009	.012 (.116)	.005
Constant	.276 ** (.071)		.212 ** (.075)		-.189 * (.088)		-.156 $\dagger$ (.088)	
R^2	.298		.310		.294		.304	
R^2 increase due to interaction			.012				.010	

Notes: Unstandardized (*b*) and standardized coefficients (β) shown with robust standard errors in parentheses; *N* = 388.

$\dagger$ *p* .10,

* *p* .05,

** *p* .01 (two-tailed tests).