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Oxytocin receptor genetic and epigenetic variation: association with child abuse and adult psychiatric symptoms

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Abstract

Childhood abuse can alter biological systems and increase risk for adult psychopathology. Epigenetic mechanisms, alterations in DNA structure that regulate the gene expression, are a potential mechanism underlying this risk. While abuse associates with methylation of certain genes, particularly those in the stress response system, no study to date has evaluated abuse and methylation of the oxytocin receptor (*OXTR*). However, studies support a role for *OXTR* in the link between abuse and adverse adult outcomes, showing that abuse can confer greater risk for psychiatric symptoms in those with specific *OXTR* genotypes. Our study therefore sought to (1) assess the role of epigenetics in the link between abuse and psychopathology and (2) to begin to integrate the genetic and epigenetic literature by exploring associations between *OXTR* genotypes and DNA CpG methylation. Data on 18 *OXTR* CpG sites, 44 SNPs, childhood abuse, and adult depression and anxiety symptoms were assessed in 393 African American adults (age = 41±12.8). Overall, 68% of genotypes associated with methylation of nearby CpG sites, with a subset surviving multiple test correction. Child abuse associated with higher methylation of two CpG sites yet did not survive correction or serve as a mediator of psychopathology. However, abuse interacted with CpG methylation to predict psychopathology. These findings suggest a role for *OXTR* in understanding the influence of early environments on adult psychiatric symptoms.

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Keywords

DNA methylation; epigenetic; OXTR; oxytocin; gene-environment interactions; child abuse

Exposure to abuse during childhood is a strong predictor of increased risk for adverse adult outcomes such as psychopathology (Mancini, Van Ameringen, & MacMillan, 1995; Bradley, Binder, & Epstein, 2008). Mechanisms by which early adversity results in these outcomes has been a topic of intense research and has been explored from different biological perspectives (Shonkoff, 2010). However, not all individuals exposed to adverse contexts will develop poor outcomes, suggesting that certain factors, such as genetic predispositions, may place individuals at varying risk (Belsky et al., 2009). Recent studies have suggested that the oxytocin system may be important to consider in these processes given the social context of abuse and the early environment.

Oxytocin is a neurohormone involved in social behaviors and the development of bonds (Bartz, Zaki, Bolger, & Ochsner, 2011; Insel & Young, 2001). Recent studies suggest that oxytocin functions to increase the salience of social cues and thus influences an individual's sensitivity to the social environment (Bartz et al., 2011). Given this, oxytocin can result in both enhanced positive or negative outcomes depending on the level of nurturance or adversity in the environment (Bartz et al., 2011). Recent gene-environment studies assessing *OXTR* SNPs (single nucleotide polymorphisms) have provided support for this concept, whereby genotypes that associate with positive behaviors such as empathy (Wu, Li, & Su, 2012) and trust (Krueger et al., 2012) also confer greater risk for depression and anxiety following adverse environments such as abuse (McQuaid, McInnis, Stead, Matheson, & Anisman, 2013; Myers et al., 2014; Thompson, Parker, Hallmayer, Waugh, & Gotlib, 2011).

In addition, early life experiences have also been found to influence the development of the oxytocin system. For example, women exposed to childhood abuse have lower levels of oxytocin in their cerebrospinal fluid (Heim, Young, Newport, & Mletzko, 2008), and rodents exposed to low levels of nurturing have lower oxytocin receptors levels in certain areas of the brain (Francis, Champagne, & Meaney, 2000). One mechanism by which these early environments can “get under the skin” and influence biological systems is epigenetics, the term applied to structural modifications that can regulate gene expression without changing the DNA sequence. One of the most commonly studied epigenetic modifications is the addition of a methyl group to DNA (Bird, 2002). DNA methylation primarily occurs at CpG sites, in which cytosine and guanine basepairs occur consecutively and are linked by phosphate bonds. When this addition occurs in the promoter region of a gene, it can block the binding of factors that regulate gene expression, and thus reduce overall expression of the gene (Klose & Bird, 2006).

Much of the excitement surrounding epigenetics stems from the ability of the environment to alter methylation patterns of certain genes, particularly those involved in the stress response system (McGowan et al., 2009; Szyf & Bick, 2012). This ability points to epigenetics as a possible mechanism by which adverse early environments can result in lasting biological changes that may increase an individual's risk for poor adult outcomes (Meaney, 2010; Szyf & Bick, 2012). Initial human studies exploring *OXTR* methylation

have identified associations between methylation and certain behaviors, such as autistic behaviors (Gregory et al., 2009) and unemotional and callous behaviors (Dadds et al., 2013). However, no study to date has evaluated *OXTR* methylation for association with the early environment or adult psychopathology. When considering the associations between the environment and *OXTR* methylation, it is important to note that the environment may influence DNA methylation to different extents across different genes. Given the lack of both human and animal studies to guide this question, our study sought to evaluate whether *OXTR* methylation may mediate or moderate the association between abuse and adult psychopathology.

To date, genetic and epigenetic risk factors have primarily been studied separately, though there are correlations between them. Initial evidence suggests that some SNPs associate with the degree of methylation of nearby CpG sites (Bell et al., 2012; Smith et al., 2014). It is therefore plausible that these associations may provide a mechanism by which certain SNPs associate with behavioral and health outcomes. Indeed DNA methylation may be the mechanism underlying some gene-environment interactions. Integrating both SNPs and DNA methylation into conceptual models of abuse and adult psychopathology will provide a more comprehensive understanding of biological processes underlying the risk for adverse outcomes following abuse.

METHOD

Participants

We evaluated subjects recruited for a larger study investigating genetic and environmental influences on post-traumatic stress disorder (Gillespie et al., 2009). Subjects were approached in waiting rooms of primary care or obstetrical-gynecological clinics of a large, urban, public hospital in Atlanta, GA. Subjects willing to participate provided written informed consent and participated in a verbal interview and blood draw. As we have previously reported (Gillespie et al., 2009), this cohort is characterized by high rates of interpersonal violence and psychosocial stress. The majority of subjects have been exposed to one major trauma during their lifetime, and the number of traumatic experiences predict psychiatric symptom severity (Binder, Bradley, Liu, & Epstein, 2008; Bradley et al., 2008). All subjects who had *OXTR* methylation data were included in the current study ($N = 393$). Subjects were an average age of 41 ± 12.8 years (range 18 – 77), the majority were female (70.7%), and all subjects were African-American and lived in urban neighborhoods of low socioeconomic status. Study procedures were approved by the Institutional Review Board of Emory University School of Medicine and the Grady Health Systems Research Oversight Committee.

Measures

Demographic information including age, sex, and race was provided on a self-administered form. The Childhood Trauma Questionnaire (CTQ), a retrospective self-report inventory, was used to assess physical, sexual, and emotional abuse during childhood based on the established scores for mild, moderate, and severe abuse for each type (Bernstein & Fink, 1998; Bernstein, Stein, Newcomb, & Walker, 2003). The CTQ was used to create a

composite score to classify each participant based on a history of any abuse (physical, sexual, or emotional), resulting in 3 categories: none or mild ($N = 200$), moderate ($N = 100$), or severe ($N = 89$). Lifetime exposure to traumatic events was assessed using the Traumatic Events Inventory (Bradley et al., 2008; Schwartz, Bradley, Sexton, Sherry, & Ressler, 2005), which measures lifetime (childhood and adulthood) exposure to trauma such as natural disaster, serious accident or injury, and physical or sexual assault. Depressive symptoms were indexed using the 21-item Beck Depression Inventory (BDI), a widely used continuous measure of depressive symptoms (Beck, Steer, & Brown, 2005). Anxiety symptoms were assessed as a continuous score from the Hamilton Anxiety Scale (HAM-A) (Maier, Buller, Philipp, & Heuser, 1988). The correlation between depression and anxiety symptoms in this cohort is 0.51 ($p < .001$).

DNA Extraction & Assays

Whole blood was collected in EDTA tubes. DNA was quantified using PicoGreen (Invitrogen) and the quality was checked on an agarose gel. Genotyping was performed using the Omni-Quad 1M or the Omni Express BeadChip (Illumina). The majority of SNPs assessed were consistent on both arrays. However, the sample size is reduced for some SNPs due to their inclusion on only one array ($95 < N < 312$). PLINK was used to perform quality control analyses such that SNPs that had a call rate $< 95\%$, a minor allele frequency (MAF) $< .05$, or significant deviation from Hardy-Weinberg proportions ($p < .00001$) were excluded, as were samples with $> 5\%$ missing data. From this data, we evaluated 44 SNPs within and near the *OXTR* gene (Supplementary Table 1).

One microgram of DNA was bisulfite-treated for cytosine to thymine conversion using the EZ DNA Methylation-Gold kit (Zymo Research). The DNA was then whole-genome amplified, fragmented, and hybridized to the HumanMethylation450 BeadChip (Illumina). The BeadChips were scanned using a BeadStation 500GX, and the methylation level (beta value) was calculated for each queried CpG locus using the Methylation Module of BeadStudio software. Beta values were set to missing (no call) if detection p-values exceeded .001. CpGassoc (Barfield, Kilaru, Smith, & Conneely, 2012) was used to remove samples with probe detection call rates $< 95\%$ and those with an average intensity value of either $< 50\%$ of the experiment-wide sample mean or $< 2,000$ arbitrary units (AU). In addition, CpG sites with missing data for $> 10\%$ of samples were excluded from analysis. Finally, probes with known SNPs or that cross hybridize between autosomes and sex chromosomes were also removed (Chen et al., 2013). No samples warranted removal. From this data, we evaluated 18 *OXTR* CpG sites. The probe sequence of one CpG site, cg00078085, contains a common SNP, rs73132856. This SNP was not evaluated in our study, and the CpG site did not associate with abuse or psychiatric symptoms.

Statistical Analysis

Data from leukocyte subtypes (GEO GSE35069) was used to identify CpG sites that are tissue-specific for certain cell types, and the method described by Houseman and colleagues was used to estimate the proportion of granulocytes and lymphocytes in our whole blood DNA samples (Houseman, Accomando, & Koestler, 2012; Koestler et al., 2013; Sun et al., 2013). Age, sex, cellular heterogeneity (percent of granulocytes and lymphocytes), and

positional effects (sample chip and row) associate with methylation of one or more *OXTR* CpG sites and were included as covariates in all analyses.

One-way ANOVAs were used to test for mean differences in covariates and psychiatric symptoms across abuse categories. The association between each SNP within or near *OXTR* (chr3:8,788,096–8,841,641; hg19) and the proportion of methylation at each CpG site was examined using regressions, where methylation was modeled as a linear function of the number of reference alleles (0, 1, or 2), adjusting for the covariates above.

To evaluate the association between child abuse and methylation, methylation of each CpG site was modeled as a linear function of abuse (none to mild, moderate or severe), adjusting for covariates. To begin to incorporate genetic-epigenetic associations into these analyses, the models were retested while including SNPs that associate with the CpG site after multiple test correction. To explore whether CpG methylation may mediate the association between abuse and psychiatric symptoms, abuse-associated CpG sites were tested for association with depression and anxiety, adjusting for covariates.

To test whether DNA methylation moderates the relation between abuse and psychiatric symptoms, an interaction term for abuse and methylation of each CpG site was included in models predicting psychiatric symptoms. For visual representation, we explored the direction of these interactions by using simple slope procedures and graphing the association between abuse and psychiatric symptoms for one standard deviation above and below the mean CpG methylation level. To integrate the genetic-epigenetic associations into these models, the interaction analyses were repeated while controlling for one of each of the SNPs that associated with the CpG site of interest after multiple test correction. Because interaction analyses can yield spurious results when the variance of a quantitative outcome differs by level of the environmental variable (Voorman et al., 2011, Almli et al., 2014), top interaction results were tested for statistical robustness by computing robust standard errors using the “sandwich” package in R (Zeileis, 2004). Finally, since studies traditionally test interactions between SNPs and the environment, we tested abuse $\times$ SNP interactions on psychiatric symptoms, adjusting for sex, and repeated these analyses controlling for associated CpG sites.

We present results for all nominal associations ($p < .05$) as a resource for investigators interested in specific SNPs or CpG sites along with the Bonferroni threshold that considers the number of independent tests. To determine the number of tests performed, we examined pairwise correlation between each CpG site and its nearest neighbors and identified significant correlations across all but one of the adjacent CpGs. Based on the direction and significance of the correlation across the gene (Supplementary Table 2), we estimate 5 methylation blocks in *OXTR*. We performed a similar analysis for the SNPs included in this study. PLINK was used to assess linkage disequilibrium (LD), suggesting 11 LD blocks in this cohort and 27 independent genetic tests. These estimates were used to calculate the appropriate number of independent tests considered for our Bonferroni correction for i) associations between SNPs and methylation (135 tests, $\alpha = 3.7 \times 10^{-4}$), ii) associations between methylation and a trait (5 tests, $\alpha = 0.01$), and iii) associations between SNPs and a trait (27 tests, $\alpha = .0019$).

RESULTS

Study Cohort

Approximately half of the subjects were exposed to physical, emotional, or sexual abuse at moderate (25.7%; $n = 100$) or severe (22.9%; $n = 89$) levels (Table 1). Across these categories, there were no differences in age or blood sample cellular proportions; however females reported a history of moderate or severe abuse during childhood at a higher rate than males ($p < .05$). Sex was included as a covariate in all models. Within the categories of moderate or severe abuse, 24.9% of the sample experienced physical abuse ($n = 97$), 23.5% experienced emotional abuse ($n = 91$), and 45% experienced sexual abuse ($n = 130$). Those who reported moderate or severe abuse also experienced greater levels of trauma over the lifespan and had higher levels of adult depression and anxiety ($p < .001$). For the *OXTR* methylation data, average methylation levels varied across CpG sites, with sites immediately upstream of the gene or within the 3rd intron tending to show higher levels of methylation compared to other sites across *OXTR* (Supplementary Table 3).

Genetic-epigenetic associations in *OXTR*

We evaluated the association between 44 genotypes and methylation of 18 *OXTR* CpG sites for all pairwise combinations (Figure 1; Table 2). Overall, the majority of SNPs associated with at least one CpG site ($N = 30$ of 44; 68.2%; $p < .05$), with an average distance of 10.5 kilobases (kb) between associated SNP-CpG pairs. Conversely, the majority of CpG sites associated with one or more SNPs ($N = 14$ of 18; 77.8%; $p < .05$). Four of these pairs survive correction for multiple testing (Table 2). For example, individuals with a GG genotype (versus AG or AA genotypes) at rs2301261 had higher DNA methylation levels at cg00247334 and cg25140571, two CpG sites in the *OXTR* promoter.

OXTR methylation as a mediator of abuse and psychiatric symptoms

A history of abuse during childhood predicted higher methylation of 2 CpG sites (cg04523291 and cg02192228) located in exon 3 of *OXTR* ($.011 < p < .017$). While these sites are in close proximity and their methylation is highly correlated ($r = 0.882$, $p < .01$), neither association survived correction for multiple testing. Furthermore, in testing a mediation model, neither CpG site predicted depression and anxiety symptoms ($p > .05$), suggesting that *OXTR* methylation does not mediate the association between abuse and psychiatric symptoms. Neither CpG site was associated with nearby SNPs when accounting for multiple test correction (Table 2), and including any of the nominally associated SNPs as covariates did not substantially change the results (data not show).

OXTR methylation as a moderator of abuse and psychiatric symptoms

Psychiatric symptoms were higher among those with abuse (Table 1) but were not directly associated with *OXTR* methylation. However, abuse interacted with methylation of multiple *OXTR* CpG sites to predict psychiatric symptoms (Table 3). Five CpG sites interacted with abuse to predict depression, and three of these also interacted to predict anxiety. Furthermore, these 3 overlapping sites survived multiple test correction for both outcomes ($p < 0.01$). The direction of the interaction was dependent upon CpG location. For example,

individuals with lower methylation of cg08535600 in exon 1, located in the promoter region of the gene, reported higher depressive ($p = 5.4 \times 10^{-4}$) and anxiety ($p = 7.5 \times 10^{-5}$) symptoms compared to those with higher methylation at this site, if they report a history of abuse (Figure 2A–B). Alternatively, those with higher methylation of cg11589699, located in the 3rd intron of the gene, report higher depressive ($p = 5.5 \times 10^{-4}$) and anxiety ($p = 7.6 \times 10^{-4}$) symptoms, compared to those with lower methylation, if they also report a history of abuse (Figure 2C–D). Methylation levels of CpG sites located in intron 3 correlate positively with each other and negatively with the sites located in exon 1 (Supplementary Table 2).

Of the five CpG sites that interact with abuse to predict depression, one site, cg00385883, associates with two SNPs after multiple test correction. When controlling for each of the two associated SNPs in abuse $\times$ methylation models, the interaction between abuse and methylation remained significant, though nominally for one ($p = .008$ for rs7629329 and $p = .022$ for rs237897; corrected $\alpha = 0.01$). Finally, because interaction analyses can produce spurious results if the variance of a quantitative outcome differs by level of the environmental variable (Voorman et al., 2011, Almli et al., 2014), we also present p-values from a robust version of the interaction test (Zeileis, 2004) and note that our results remain consistent (Table 3).

Integration of *OXTR* SNPs and methylation

Traditionally, studies evaluate the interaction of SNPs and an environmental exposure; we identified 6 SNPs that interact with abuse to predict psychiatric symptoms (Supplementary Table 4). Interpretation should proceed with caution given the number of tests, as none remained significant after multiple test correction (27 independent tests for each outcome, $\alpha = .0019$). Two of the SNPs (rs9817913 and rs237852) do not associate with any CpG sites in our study, and the other 4 (rs237837, rs3901926, rs9860869, rs237889) do not remain associated with any study CpG sites after multiple test correction. However, inclusion of nominally associated CpG sites in the models did not substantially change the abuse $\times$ SNP interaction results (data not shown). While rs3901926 and rs237889 interacted with abuse to predict psychiatric symptoms, their associated CpG sites did not interact with abuse. However, rs9860869 interacted with abuse to predict anxiety, and methylation of its associated CpG site (cg02192228) was directly associated with abuse. Finally, rs237837 interacted with abuse to predict anxiety, and its associated CpG site (cg11589699) interacted with abuse to predict both depression and anxiety.

DISCUSSION

Our study explored the role of *OXTR* methylation in the link between abuse during childhood and adult psychopathology. We found that abuse associates with higher methylation at two CpG sites, both located within exon 3 of the gene. However, this finding did not reach significance when accounting for multiple testing nor did these sites serve as a mediator of psychopathology. This finding is intriguing given the relative lack of human and animal studies assessing this question to date, despite the rapidly growing literature on the role of the early environment on methylation of a number of genes (Szyf & Bick, 2012; McGowan et al., 2009). However, it is plausible that *OXTR* methylation may still be

responsive to the environment. A recent study found that stress may be able to influence small rapid changes in *OXTR* methylation. Unternaehrer et al. (2012) investigated dynamic changes in CpG methylation across the majority of *OXTR* exon 3 and found that average methylation increased following the Trier Social Stress Test (TSST) and then decreased below pre-TSST levels 90-min post-test. While the influence of stress on methylation of other regions of the gene was not explored, it is of interest that this segment, which overlaps with the span containing CpG sites nominally associated with abuse in our study, may dynamically change in response to the environment. Of question is the degree to which methylation of this site may be influenced by the environment, and whether methylation may become fixed following stress during developmentally sensitive time-periods, or may continue to respond to stress over time. For example, it is possible that our nominal associations may be more representative of exposure to current stressful environments. Future research should incorporate thorough assessments of both early and concurrent stress to begin to address this question and current limitations.

While *OXTR* CpG methylation did not serve as a mediator to psychiatric symptoms, we did find that it served as a moderator for abuse and psychiatric symptoms. Abuse interacted with methylation at specific *OXTR* CpG sites to predict depression and anxiety. Three of the five CpG sites that interacted with abuse to predict depression survived Bonferroni correction, and these were the same three sites found to predicted anxiety outcomes. Interestingly, the pattern of the interaction differed depending on the area of the gene where the CpGs were located. For CpG sites within exon 1, part of the *OXTR* promoter region, individuals with lower levels of methylation reported higher depression and anxiety if they reported abuse. For sites located in intron 3, within the gene body, an opposite pattern was observed – individuals with higher methylation reported higher depression and anxiety if they reported abuse. Methylation at these sites grouped together; the significant sites in exon 1 were correlated with each other and negatively correlated with sites in intron 3. Individuals who tended to have lower methylation of the CpG sites in exon 1 also tended to have higher methylation in intron 3 and, in our study, lower methylation of the CpG sites within exon 1, and higher methylation of the CpG sites within intron 3, associated with greater risk for psychopathology following abuse. To date, the function of the these CpG sites is still unknown. Methylation of promoter regions is often conceptualized as reducing gene expression, though the role of gene body methylation is relatively unclear and high gene body methylation has been reported for genes that are highly expressed (Maunakea et al., 2010). Previous reports have suggested that methylation across *OXTR* may associate with *OXTR* regulation and expression (Harony-Nicolas et al., 2014; Mamrut et al., 2013; Mizumoto et al., 1997); however, additional research will be important for interpretation of these findings.

Exploring the interplay between genetics, epigenetics and the early environment is relatively new, yet it has the potential to provide additional insight into the development of later outcomes. When incorporating epigenetics into conceptual models of complex behavioral outcomes, an initial question to address is how to integrate genetic and epigenetic variation. Our study identified a number of associations between *OXTR* SNPs and DNA methylation of specific CpG sites. On average, SNPs were a moderate distance from their associated

CpG sites (~10.5 kb). This is consistent with patterns observed across the genome (Smith et al., 2014), and suggests that DNA methylation may provide a functional link between genetic variation and gene expression (Bell et al., 2012). In this study, we observed both individual SNPs and DNA methylation interacting with abuse to predict psychiatric symptoms. In some cases, genetic or epigenetic variation associated independently, though there was also overlap between SNPs that interact with abuse and CpG sites that associate with abuse. This may suggest a potential mechanistic link underlying some $G \times E$ findings, yet continues to allow for additional mechanisms underlying these associations.

Three SNPs associate with CpG sites after correction for multiple tests (rs2301261, rs237897 and rs7629329). These SNPs have been examined in the *OXTR* literature previously, and one of these SNPs, rs7629329, associated with expression of *OXTR* in human brain tissue (Myers et al., 2014), which may suggest a possible role of methylation in this finding. However, the majority of reports, which primarily assess SNP associations with autistic or social behaviors, have not found associations with these SNPs (Campbell et al., 2011; Wu et al., 2012), and few studies have assessed interactions between these SNPs and early adversity (Loth et al., 2013; Myers et al., 2014). It is possible that the literature has not evaluated behaviors important for these SNPs or their associated CpG sites. It is also possible these SNP-CpG associations may be specific to our sample or to the African American population. Lastly, the functional significance of these SNPs and their associated CpG sites in regulating *OXTR* is not well understood.

Two of the most commonly studied *OXTR* SNPs are rs53576 and rs2254298. The mechanism through which these SNPs may influence behavioral outcomes has yet to be elucidated. We identified one associated CpG site for each SNP, though our sample size was limited for these analyses ($N \sim 100$). While these SNPs did not interact with abuse to predict psychiatric symptoms in our cohort ($p > .05$), the CpG sites associated with these SNPs did interact with abuse to predict adult psychiatric symptoms. Individuals with the G allele of rs53576 had higher methylation at cg00385883, and individuals with higher methylation at this site reported nominally higher depression symptoms if they reported childhood abuse. McQuaid et al. (2013) recently reported that individuals with the rs53576 G allele had higher depressive symptoms following exposure to childhood maltreatment. For rs2254298, individuals with the G allele had higher methylation at cg11589699, and higher methylation at this site was found to associate with both increased depression and anxiety symptoms among those reporting abuse. The rs2254298 GG genotype has previously been associated with both unipolar depression and adult attachment anxiety (Costa et al., 2009), and Thompson and colleagues reported an interaction between this SNP and early adversity (conceptualized as a mother with major depression), though the AG genotype was associated with the highest depression and anxiety outcomes (Thompson et al., 2011). It may be possible that rs53576 and rs2254298 associate with psychiatric outcomes through *OXTR* methylation. Replication and further research exploring this possibility are warranted.

Limitations

While this study provides new insight into the interplay between genetic and epigenetic variation in *OXTR*, the biological consequences of childhood abuse, and the interaction

between abuse and methylation on adult psychiatric symptoms, there are a number of important limitations to address. First, childhood abuse was assessed retrospectively with a self-report measure that does not include detailed information on the abuse and its psychological impact. However, the CTQ is a well-validated measure that has been used previously in the literature to assess abuse during childhood. Additional insight will likely be gained by including a more detailed assessment of abuse timing and type on the development of biological changes and adverse outcomes.

Second, we sought to test associations between events in early life and DNA methylation in adulthood. The degree to which methylation remains fixed following sensitive developmental time periods, or continues to change in response to the environment, is still a topic of debate and is not fully known. While retrospective cross-sectional samples can begin to highlight genes that may be important for investigating the question of timing, prospective longitudinal studies will be critical for studying how stressful experiences may influence epigenetic programming over time.

Sample size is an important concern for both genetic and epigenetic studies, and our sample size is limited, particularly for the subsample with genotype data for rs53576 and rs2254298, two of the most commonly studied *OXTR* SNPs. Therefore, associations should be considered preliminary prior to replication. However, for methylation analyses, our sample is considerably larger than any previous studies of *OXTR* methylation, which often have less than 100 participants.

The majority of human *OXTR* methylation studies to date have assessed methylation of sites within the first intron of the gene (Kumsta et al., 2013). Our study includes data on two CpG sites within the first intron, though comparability between previous findings and our study is limited given different areas covered. Future work should continue to assess CpG sites previously reported as well as those across the gene, particularly in the promoter and intron 3, to provide a more comprehensive understanding of *OXTR*.

The range and variance of methylation differs across CpG sites in this gene. While the functional significance of this is not known, it may have implications for statistical testing and interpretation as CpG sites with low variance will have attenuated covariance with other variables and a reduced likelihood of achieving association. Furthermore, when interpreting high versus low methylation of specific CpG sites, absolute levels of high and low methylation will be limited by the range and average methylation of CpG site of interest, and can differ from the levels of other CpG sites.

Our study was limited to utilizing peripheral tissue. *OXTR* methylation should ideally be assessed in the tissues that are known to express *OXTR* and directly involved in psychiatric symptoms. While initial post-mortem studies have reported *OXTR* methylation and expression differences in the brain tissue of those with and without autism (Gregory et al., 2009), no studies to date have looked at associations between abuse and *OXTR* methylation in human brain tissue, though differences by abuse exposure have been reported for other genes (McGowan et al., 2009). The degree to which methylation of peripheral tissues can be used to study methylation changes in response to the environment or in association with

behavioral outcomes is currently a topic of debate, though there is some evidence that methylation in peripheral tissues may be comparable to that of brain (Smith et al., 2014). Further understanding will be paramount for interpreting and conducting human methylation studies given this important yet practical limitation.

Lastly, our study did not evaluate gene expression and thus cannot for the role of study CpG sites on regulation and expression. Previous research has shown functional significance of areas within the *OXTR* promoter region and intron 3 (Kumsta et al., 2013). Furthermore, a recent study reported that a number of *OXTR* SNPs associate with expression level in human brain tissue (Myers et al., 2014). Eight of the SNPs were present in our dataset, and all of these SNPs associate with methylation of a nearby CpG site in our sample ($1.0 \times 10^{-4} < p < .046$), which may point to a possible role of methylation in this finding and warrants further research.

Conclusions

Interest in the role of genetic and epigenetic influences on behavioral outcomes has grown quickly, with studies calling for the inclusion of *OXTR* (Kumsta et al., 2013). Our study identified nominally higher methylation of two *OXTR* CpG sites among those reporting abuse, and found that CpG methylation may interact with abuse to predict psychiatric outcomes. Furthermore, significant SNP-CpG associations were found, supporting a potential mechanism by which some SNPs may associate with gene expression. Given oxytocin's important role in social behavior, research on *OXTR* genetics and epigenetics will continue to provide important insight into how the oxytonergic system may underlie and influence behavioral development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *OXTR* structure and variant location

The oxytocin receptor, located on chromosome 3p25, has four exons and three introns that span 17kb. Only SNPs and CpG sites assessed in this study are depicted. Table 2 provides a key for CpG abbreviations, and Supplementary Table 1 provides a key for SNP labels.

Figure 2. *OXTR* methylation moderates the association between childhood abuse and adult psychiatric symptoms

These figures display the pattern of interaction between abuse and CpG site methylation for CpG sites that associate with both depression and anxiety after Bonferroni correction. (A) Association between abuse and depression for one standard deviation above and below the mean methylation of cg08535600, located in exon 1. (B) Association between abuse and anxiety for one standard deviation above and below the mean methylation of cg08535600. (C) Association between abuse and depression for one standard deviation above and below the mean methylation of cg11589699, located in intron 3. (D) Association between abuse and anxiety for one standard deviation above and below the mean methylation of cg11589699.

Table 1

Study Variables Among those with None to Mild, Moderate, or Severe Abuse

	Physical, Emotional, or Sexual Abuse			p-value
	None to Mild (N=200)	Moderate (N=100)	Severe (N=89)	
Age (Mean, SD)	41.3 (13.2)	40.1 (13.2)	42.4 (11.3)	0.46
Females (N, %)	130 (65.0%)	77 (77.0%)	68 (76.4%)	0.040
% Granulocytes	61.9 (10.2)	62.2 (12.4)	62.1 (10.9)	0.89
% Lymphocytes	15.0 (5.8)	15.1 (8.3)	14.2 (5.0)	0.56
Lifetime Trauma	3.8 (2.9)	5.5 (2.8)	8.1 (3.4)	<.001
BDI Score	12.1 (10.5)	16.4 (11.6)	23.5 (14.0)	<.001
HAM-A Score	8.8 (7.9)	11.1 (8.2)	15.1 (9.9)	<.001

Note: Percent granulocyte and lymphocyte represents the percent of that cell type in the blood sample used for methylation analyses, and represents the cellular heterogeneity within the sample. Lifetime Trauma is the number of traumatic events experienced or witnessed upon time of study participation. Sample range was 0–16. BDI is the Beck Depression Inventory and HAM-A is the Hamilton Anxiety Rating Scale. These scales range from 0–58 for BDI and 0–37 for HAM-A, with higher scores represent higher depression or anxiety symptoms, respectively.

Table 2
Association between Single Nucleotide Polymorphisms and Methylation of Specific CpG Sites

			Model Statistics				SNP Predictor Statistics			
CpG site	CpG position	SNP	SNP position	R-squared	F statistic	p-value	Beta	t-statistic	p-value	
cg14483142	8811758 (r)	Not associated with any tested SNPs								
cg17036624	8811601 (q)	Not associated with any tested SNPs								
cg00247334	8811543 (p)	rs2301261	8810896 (29)	0.089	(7, 310) = 4.23	1.8×10^{-4}	-0.24	-4.27	2.6×10^{-5} *	
		rs6777726	8813494 (30)	0.066	(7, 309) = 3.03	0.004	-0.18	-3.20	0.002	
		rs237888	8797095 (15)	0.060	(7, 310) = 2.76	0.009	0.16	2.90	0.004	
		rs2268495	8807535 (24)	0.054	(7, 309) = 2.45	0.019	0.15	2.61	0.010	
		rs237899	8808515 (26)	0.054	(7, 310) = 2.49	0.017	-0.14	-2.55	0.011	
		rs237902	8809184 (27)	0.171	(7, 94) = 2.56	0.019	0.23	2.32	0.023	
cg25140571	8811437 (o)	rs2301261	8810896 (29)	0.093	(7, 310) = 4.46	9.6×10^{-5}	-0.22	-4.02	7.5×10^{-5} *	
		rs3901926	8834933 (42)	0.068	(7, 309) = 3.17	0.003	0.15	2.74	0.006	
		rs237888	8797095 (15)	0.064	(7, 310) = 2.95	0.005	0.14	2.45	0.015	
		rs2139184	8795494 (10)	0.060	(7, 309) = 2.78	0.008	-0.12	-2.19	0.029	
		rs237885	8795543 (11)	0.059	(7, 310) = 2.70	0.010	0.12	2.08	0.038	
		rs918316	8798181 (16)	0.059	(7, 310) = 2.70	0.010	-0.12	-2.08	0.038	
cg23391006	8811279 (n)	rs237899	8808515 (26)	0.059	(7, 310) = 2.70	0.010	-0.12	-2.08	0.039	
		rs11476	8788198 (2)	0.274	(7, 94) = 4.70	1.7×10^{-4}	0.27	2.85	0.005	
cg09353063	8811092 (m)	Not associated with any tested SNPs								
cg17285225	8811004 (l)	rs11131149	8802851 (21)	0.220	(7, 309) = 12.18	$<1.0 \times 10^{-6}$	-0.13	-2.42	0.016	
		rs6443206	8820075 (33)	0.275	(7, 94) = 4.71	1.6×10^{-4}	0.22	2.34	0.022	
cg085535600	8810980 (k)	rs237895	8807423 (23)	0.251	(7, 94) = 4.16	0.001	-0.3	-3.11	0.003	
cg00078085	8810592 (j)	rs237889	8802483 (20)	0.059	(7, 309) = 2.71	0.010	-0.13	-2.37	0.018	
		rs2268495	8807535 (24)	0.056	(7, 308) = 2.53	0.015	-0.12	-2.11	0.036	
cg03987506	8810549 (i)	rs237885	8795543 (11)	0.055	(7, 309) = 2.52	0.016	-0.12	-2.08	0.038	
		rs6793234	8826396 (37)	0.054	(7, 309) = 2.45	0.019	0.11	1.98	0.049	
cg19619174	8810139 (h)	Not associated with any tested SNPs								
cg12695586	8810077 (g)	rs11476	8788198 (2)	0.260	(7, 94) = 4.37	3.4×10^{-4}	0.33	3.46	0.001	
		rs237899	8808515 (26)	0.080	(7, 310) = 3.77	0.001	-0.18	-3.18	0.002	

CpG site	CpG position	SNP	SNP position	Model Statistics			SNP Predictor Statistics		
				R-squared	F statistic	p-value	Beta	t-statistic	p-value
cg27501759	8809715 (f)	rs7628723	8832470 (40)	0.223	(7, 94) = 3.56	0.002	-0.25	-2.57	0.012
		rs11476	8788198 (2)	0.212	(7, 94) = 3.34	0.003	0.23	2.31	0.023
		rs237925	8821742 (36)	0.065	(7, 310) = 3.02	0.004	0.13	2.26	0.025
		rs7629329	8788336 (3)	0.061	(7, 309) = 2.80	0.008	-0.12	-2.11	0.036
		rs7632031	8818757 (32)	0.063	(7, 310) = 2.92	0.006	0.12	2.11	0.036
		rs237925	8821742 (36)	0.087	(7, 310) = 4.14	2.3×10 ⁻⁴	0.15	2.70	0.007
cg02192228	8809536 (e)	rs237897	8808285 (25)	0.083	(7, 215) = 2.69	0.011	0.17	2.53	0.012
		rs6443206	8820075 (33)	0.170	(7, 94) = 2.54	0.020	0.20	2.03	0.045
		rs237925	8821742 (36)	0.068	(7, 310) = 3.14	0.003	0.19	3.42	0.001
		rs180789	8813927 (31)	0.058	(7, 310) = 2.65	0.011	-0.16	-2.90	0.004
		rs9860869	8820740 (35)	0.048	(7, 310) = 2.16	0.037	0.13	2.25	0.025
		rs6791619	8829872 (39)	0.046	(7, 307) = 2.06	0.047	-0.13	-2.23	0.026
cg04523291	8809501 (d)	rs237925	8821742 (36)	0.057	(7, 310) = 2.59	0.013	0.18	3.15	0.002
		rs180789	8813927 (31)	0.046	(7, 310) = 2.10	0.043	-0.15	-2.57	0.011
cg15317815	8809306 (c)	rs237925	8821742 (36)	0.051	(7, 310) = 2.34	0.025	0.19	3.33	0.001
		rs237897	8808285 (25)	0.170	(7, 215) = 6.11	2.0×10 ⁻⁶	-0.29	-4.52	1.0×10 ⁻⁵ *
cg00385883	8808259 (b)	rs7629329	8788336 (3)	0.124	(7, 309) = 6.13	1.0×10 ⁻⁶	0.21	3.94	1.0×10 ⁻⁴ *
		rs237899	8808515 (26)	0.115	(7, 310) = 5.65	4.0×10 ⁻⁶	0.19	3.57	4.1×10 ⁻⁴
		rs13093809	8788096 (1)	0.105	(7, 310) = 5.07	1.8×10 ⁻⁵	0.17	3.01	0.003
		rs237895	8807423 (23)	0.179	(7, 94) = 2.71	0.014	0.31	3.03	0.003
		rs53576	8804371 (22)	0.242	(7, 100) = 4.23	4.3×10 ⁻⁴	-0.21	-2.27	0.025
		rs2301261	8810896 (29)	0.093	(7, 310) = 4.44	1.0×10 ⁻⁴	0.12	2.22	0.027
		rs75775	8820732 (34)	0.151	(7, 310) = 7.70	<1.0×10 ⁻⁶	-0.17	-3.23	0.001
		rs2268495	8807535 (24)	0.151	(7, 309) = 7.70	<1.0×10 ⁻⁶	0.16	3.06	0.002
		rs6791619	8829872 (39)	0.146	(7, 307) = 7.31	<1.0×10 ⁻⁶	0.15	2.70	0.007
		rs237837	8841641 (44)	0.142	(7, 310) = 7.18	<1.0×10 ⁻⁶	0.14	2.69	0.008
cg11589699	8806317 (a)	rs237899	8808515 (26)	0.139	(7, 310) = 7.00	<1.0×10 ⁻⁶	-0.13	-2.48	0.014
		rs2301261	8810896 (29)	0.138	(7, 310) = 6.95	<1.0×10 ⁻⁶	-0.13	-2.41	0.016
		rs237911	8810008 (28)	0.231	(7, 94) = 3.72	0.001	-0.24	-2.38	0.019

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CpG site	CpG position	SNP	SNP position	Model Statistics			SNP Predictor Statistics		
				R-squared	F statistic	p-value	Beta	t-statistic	p-value
		rs2254298	8802228 (19)	0.221	(7, 94) = 3.52	0.002	-0.21	-2.12	0.037
		rs11476	8788198 (2)	0.217	(7, 94) = 3.45	0.003	-0.20	-2.02	0.046

For each CpG site, associations are shown if both the overall model and the SNP predictor reach $p < .05$. The table is organized by CpG position, starting with those upstream of the gene (larger values given its location on the negative DNA strand), and provides the actual position of each CpG (and SNP) followed in parentheses by the position label for Figure 1. Note that cg00078085 has a SNP, rs73132856, in its probe sequence. There were 135 tests in total (27 independent SNPs for each of 5 regions of methylation), leading to a Bonferroni corrected p-value of $p < 3.7 \times 10^{-4}$.

* indicates p-values maintaining significance after multiple test correction. All analyses above controlled for age, sex, cellular heterogeneity, and positional effects (sample chip and row). Standardized betas are presented. Note that sample size varies based on SNP inclusion from two genotyping arrays used in the study; sample size for each analysis can be determined by adding 1 to the second degrees of freedom in the F-statistic.

Table 3

Association between Childhood Abuse and Depression and Anxiety Symptoms: Potential Moderating Role of *OXTR* CpG site Methylation

Abuse×CpG Interaction Model	Depression			Anxiety			Methylation level with Stronger Association			
	Beta	t-statistic	p-value	p-robust	Beta	t-statistic	p-value	p-robust	Depression	Anxiety
cg23391006 (n)	-0.90	-2.28	0.023	0.028	--	--	0.67	0.69	Lower	--
cg17285225 (l)	-0.50	-3.16	1.7×10 ⁻³ *	2.1×10 ⁻³	-0.48	-2.80	0.005*	0.001	Lower	Lower
cg08535600 (k)	-0.56	-3.49	5.4×10 ⁻⁴ *	1.3×10 ⁻³	-0.70	-4.01	7.5×10 ⁻⁵ *	1.2×10 ⁻⁶	Lower	Lower
cg00385883+ (b)	6.70	2.54	0.012	0.016	--	--	0.10	0.09	Higher	--
cg11589699 (a)	6.28	3.49	5.5×10 ⁻⁴ *	9.8×10 ⁻⁴	6.58	3.40	7.6×10 ⁻⁴ *	4.8×10 ⁻⁵	Higher	Higher

Note: The interaction between abuse and CpG site methylation was tested in a linear regression predicting depression or anxiety symptoms controlling for age, sex, cellular heterogeneity, and positional effects. Models are shown if both the interaction variable (abuse x CpG methylation) and the overall model reach $p < .05$. The table is organized by CpG position, start with those more upstream in the gene, with the position label for Figure 1 provided in parentheses after the cgID. The first three CpG sites are within the exon 1, the last two are within intron 3. Standardized beta values are presented.

* indicates interactions maintaining significance after multiple test correction ($\alpha = 0.01$). "p-robust" indicates p-values estimated via a robust sandwich estimator. The direction of the interaction was explored using simple slope procedures assessing the association between abuse and psychiatric symptoms for one standard deviation above and below the mean methylation level of the associated CpG site. The final columns depict whether abuse associates with greater depression or anxiety outcomes among those that are one standard deviation above (Higher) or below (Lower) the mean methylation for the respective CpG.

+ Two SNPs associate with cg00385883 after multiple test correction. When controlling for each of these SNPs in abuse x methylation models, the interaction between abuse and methylation remained significant.