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ARTICLE

Methylation in *OTX2* and related genes, maltreatment, and depression in children

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Through unbiased transcriptomics and multiple molecular tools, transient downregulation of the Orthodenticle homeobox 2 (*OTX2*) gene was recently causatively associated with the development of depressive-like behaviors in a mouse model of early life stress. The analyses presented in this manuscript test the translational applicability of these findings by examining peripheral markers of methylation of *OTX2* and *OTX2*-regulated genes in relation to measures of depression and resting-state functional connectivity data collected as part of a larger study examining risk and resilience in maltreated children. The sample included 157 children between the ages of 8 and 15 years ($\chi = 11.4$, SD = 1.9). DNA specimens were derived from saliva samples and processed using the Illumina 450 K beadchip. A subset of children ($N = 47$) with DNA specimens also had resting-state functional MRI data. After controlling for demographic factors, cell heterogeneity, and three principal components, maltreatment history and methylation in *OTX2* significantly predicted depression in the children. In terms of the imaging data, increased *OTX2* methylation was found to be associated with increased functional connectivity between the right vmPFC and bilateral regions of the medial frontal cortex and the cingulate, including the subcallosal gyrus, frontal pole, and paracingulate gyrus—key structures implicated in depression. Mouse models of early stress hold significant promise in helping to unravel the mechanisms by which child adversity confers risk for psychopathology, with data presented in this manuscript supporting a potential role for *OTX2* and *OTX2*-related (e.g., *WNT1*, *PAX6*) genes in the pathophysiology of stress-related depressive disorders in children.

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INTRODUCTION

Using unbiased transcriptomics, transient downregulation of the Orthodenticle homeobox 2 (*Otx2*) gene in the ventral tegmental area (VTA), a key reward circuit brain region, was identified as a key molecule in the development of depressive-like behaviors in a mouse model of early life stress [1]. The transient downregulation of *Otx2* following early life stress was also associated with persistent downregulation of several target genes, many of which are involved in brain development. In this model, animals subjected to early life stress were found to exhibit exaggerated "depressive-like" behaviors after exposure to a second hit of stress in adulthood. The putative role of the transient downregulation of *Otx2* following early life stress in promoting enhanced sensitivity to subsequent stress was demonstrated by utilizing a Herpes simplex virus vector to transiently rescue *Otx2* levels immediately after early life stress. This prevented later susceptibility to stress, as did *Otx2* overexpression in adult animals. Conversely, a transient, local knockdown of *Otx2* in VTA in early life was found to mimic the effects of early life stress [1].

Otx2 is a highly conserved molecule. The two protein isoforms *Otx2* encodes are identical in both mice and humans, and functionally indistinguishable in vitro [2]. *Otx2* has multiple known roles in embryonic and experience-dependent brain development. Specifically, *Otx2* regulates genetic networks directing the specification of dopaminergic and serotonergic neurons during embryonic development [3], and in the adult brain, *Otx2* regulates neurogenesis of dopamine neurons in the VTA [4–6]. *Otx2* is also a key protein in mediating experience-dependent plasticity and defining periods of sensitive development in the visual and auditory cortices [7–9]. Although the OTX2 protein is found in the VTA, medial prefrontal cortex (mPFC), and multiple additional cortical regions [10], a similar essential role for *Otx2* in defining sensitive windows of development of other neural systems has not yet been established [8]. Given the role of *OTX2* in defining sensitive windows of development, *OTX2* has been proposed to be a potential "master-regulator" of plasticity, with likely important targets for treating stress-related psychiatric disorders [2].

To test the translational applicability of the mouse model findings and the potential utility of *OTX2* as a biomarker of stress-related psychopathology, in this paper we examine peripheral markers of *OTX2* gene methylation to determine if *OTX2* gene methylation values are associated with individual differences on

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measures of depression in children, and individual differences in connectivity in reward circuit-related regions where *OTX2* is expressed (e.g., VTA, mPFC). The relationship between *OTX2* gene methylation, measures of depression, and methylation in genes downregulated by *OTX2* are also explored.

METHODS AND MATERIALS

Subjects

The children in this study were participants in a larger study examining risk and resilience in maltreated children. The sample included 157 children from 117 families. In the year prior to study enrolment, 34% of children had an out-of-home placement due to recent reports of abuse or neglect. Another 18% of children had prior allegations of maltreatment, but were not removed, as it was determined that they could be safely maintained at home, and 48% of children were never referred to protective services. The children were between the ages of 8 and 15 years of age ($\chi = 11.4$, SD = 1.9), with the clinical and demographic characteristics of the sample depicted in Table 1.

Procedure

Assessments from parents were collected during home visits, and measures from children were collected both during home visits and during a 1-week day camp program devised specifically for our research purposes, replicating a research methodology used in prior studies [11–13]. The University of Vermont, Johns Hopkins, and Yale University Institutional Review Boards (IRB) approved the conduct of this research, with the State of Vermont Department of Children and Family Services allowing for participation of children in their custody based on the University of Vermont IRB's approval. Non-maltreated children were recruited from the community through newspaper ads and included based on parent report of an absence of significant childhood adversities, which was verified via queries of state child protective services reports of maltreatment and administration of standardized trauma screening questionnaires [14, 15]. Prior to the recruitment of maltreated children, an independent child advocate reviewed each case referred through protective services to determine that research participation was in the child's best interest. The child's parent or legal guardian provided informed consent, and each child provided assent for study participation. Birth parent assent for children in state custody was obtained when clinically appropriate (e.g., ongoing parent–child contact).

Childhood adversity and maltreatment experiences

Extending methods previously published by our team [16], multiple informants and data sources (e.g., parents, children, and protective services case records) were used to obtain a best estimate of each child's adverse childhood experiences [14–16], with the Yale–Vermont Adverse Childhood Experiences Scale (Y-VACS) clinician rating used to integrate the data from these various sources to create dimensional ratings of children's adverse life experiences [17]. The Y-VACS rates 20 types of adverse experiences, both intrafamilial (e.g., multiple forms of abuse, neglect, loss, parental incarceration, etc.) and extrafamilial (e.g., natural disasters, bullying, community violence, etc.) experiences. Each adversity receives both a frequency and severity rating. Scores generated with Y-VACS have high inter-rater reliability and strong discriminant and convergent validity [17]. In terms of maltreatment experiences, among children with a referral to protective services and no history of out-of-home placement, 58% had a history of neglect, 39% had a history of physical abuse, 19% had a history of sexual abuse, and 45% witnessed domestic violence. Among children with recent removal, 65% had a history of neglect, 67% had a history of physical abuse, 50% had a history of sexual abuse, and 70% witnessed domestic violence. A significant number of children experienced multiple adversities throughout their childhoods. The Y-VACS scores of the cohort are included in Table 1. As children with and without prior protective services involvement did not differ on the extrafamilial adversity scale, children's scores on the intrafamilial adversity scale of Y-VACS were used in subsequent analyses with methylation data to predict children's depression scores.

Depression

The Mood and Feelings Questionnaire (MFQ) [18], which is a 33-item self-report measure with excellent psychometric properties,

Table 1. Demographic and clinical characteristics of the sample ($N = 157$)

	History of maltreatment and recent placement $N = 54$	Referral to protective services, no removal $N = 29$	No history of abuse or neglect $N = 74$	Statistic *p*-value
Age	11.9 ± 2.0	11.1 ± 1.8	11.2 ± 1.9	F (2) = 2.33 p = ns
Sex (%female/%male)	59%/41%	64%/36%	54%/46%	$\chi^2 = 0.9$ p = ns
Race (EA/AA or Biracial)	90%/10%	96%/4%	85%/15%	$\chi^2 = 2.5$ p = ns
Y-VACS Intra-familial Adversity Score	27.5 ± 7.9[a]	21.2 ± 8.3[b]	9.2 ± 7.4[c]	Wald $\chi^2 = 181.5$ $p < .0001$
Y-VACS Extra-familial Adversity Score	6.0 ± 3.6	5.8 ± 3.3	5.4 ± 3.7	Wald $\chi^2 = 0.87$ p = ns
Y-VACS Total Adversity Score	33.5 ± 8.7[a]	26.9 ± 9.7[b]	14.7 ± 9.2[c]	Wald $\chi^2 = 140.6$ $p < .0001$
Mood and Feelings Depression Questionnaire	15.1 ± 12.0[a]	12.8 ± 9.8[a, b]	8.7 ± 7.2[b]	Wald $\chi^2 = 17.6$ $p = .001$
Clinical Threshold MFQ Scores	9 (17%)	4 (14%)	2 (2.7%)	$\chi^2 = 6.57^+$ $p < .04$

Means with different superscripts are statistically different from one another, Student–Newman–Keuls test
EA European American, *AA* African American, *Y-VACS* Yale–Vermont Adverse Childhood Experiences Scale, *MFQ* Mood and Feelings Questionnaire
[+]Likelihood ratio used to test the significance due to number of cells with fewer than five counts

was used to assess children's depressive symptomatology. Children's scores on the MFQ ranged from 0 to 51, with 12% of children above the clinical threshold, suggesting a likely major depression diagnosis (Table 1).

DNA specimens

DNA was extracted from the saliva samples collected while the children attended the research summer day camp program using Oragene kits, with researchers wearing protective gloves to prevent specimen contamination. Genomic DNA was extracted from the saliva using the Oragene DNA Extraction Kit (DNA Genotek, Ottawa, Ontario, Canada). To prepare specimens for methylation analysis, 500 ng of genomic DNA was treated with bisulfite reagents included in the EZ-96 DNA methylation kit (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. Bisulfite-converted DNA samples were used in the array-based genome-wide DNA methylation assay.

Array-based genome-wide DNA methylation

Illumina 450 K Methylation BeadChip interrogates > 450,000 methylation sites per sample at single-nucleotide resolution. The methylation analyses were completed at the Keck Biotechnology Resource Laboratory at Yale University. GenomeStudio software (Illumina, San Diego, CA) was used to generate β values for each CpG site, with β values ranging from 0.0 to 1.0. Raw scanned data were normalized and the average β values were recalculated using background intensity measured by negative background probes present on the array. Standard quality control tests and functional normalization were conducted using the "Preprocessfunnorm" function in the R minfi package [19]. CpG sites with detection $p > .001$ were removed to ensure that only high-confidence probes were included in subsequent analysis (2704 of 485,512 probes were removed, 0.56% of sites).

OTX2 CpG sites and OTX2-regulated genes CpG sites

We examined DNA methylation of *OTX2* and six genes regulated by OTX2 and early life stress in the mouse model. The Illumina 450K chip contains 31 CpG sites in the *OTX2* gene. The majority are in the promotor area, eight transcription start site (TSS) affiliated, 19 5′UTR associated, and four in the gene body. The BeadChip contains 203 CpG sites in the *OTX2*-regulated genes; all were analyzed, except six (3%), which failed to pass the quality control tests. The sites analyzed include 16 sites in Collagen Type VIII Alpha 1 (*COL8A1;* all gene body affiliated), 105 sites in Paired Box 6 (*PAX6;* 52 in the gene body, 43 5′UTR affiliated, and 10 TSS associated), 15 sites in Retinol-Binding Protein 3 (*RBP3;* 7 in the first exon, 4 TSS affiliated, 3 in the gene body, and one 3′UTR associated), 20 sites in Semaphorin-3C (*SEMA3C;*11 in the gene body, six TSS affilated, two 5′UTR associated, and one 3′UTR associated), 18 sites in Thrombospondin-4 (*THBS4;* nine in the gene body, seven TSS affilated, and two 5′UTR associated), and 23 sites in WNT Family Member 1 (*WNT1;* 11 TSS affilated, nine in the gene body, two 3′UTR associated, and one in the first exon).

Negative control genes

For negative control comparisons, associations with depression were examined with genes that determine height [20], as maltreatment has not been associated with changes in height. Additional requirements for negative control genes included: not associated with obesity, a highly replicated outcome associated with child maltreatment [21]; not sex-linked, as both boys and girls were included in the study, not an identified risk gene for major depression, and not associated with maltreatment-related methylation differences in our prior whole epigenome study [22]. Height-related genes examined for negative control purposes included: Abhydrolase domain containing 2 (*ABHD2*); *FLJ42289;* hyaluronan binding protein 4 (*HABP4)*; tudor domain containing 7 (*TDRD7)*; thiosulfate sulfurtransferase (rhodanese)-like domain containing 2 (*TSTD2)*; and tetratricopeptide repeat domain 23 (*TTC23)*.

Targeted bisulfite sequencing

Methylation values attained with the 450 K array were validated with targeted bisulfite sequencing. Sodium bisulfite modification was performed on 500 ng of genomic DNA. Nested polymerase chain reaction (PCR) amplification was performed prior to pyrosequencing analysis using primers described in Supplementary eTable 1, which were generated to validate the methylation values of the CpG sites used in the *OTX2, PAX6, and WNT1* genes used in the primary analyses, plus two other genes of interest to our group. After agarose gel electrophoresis, to ensure successful amplification and specificity, PCR amplicons were processed for pyrosequencing analysis according to the manufacturer's standard protocol (Qiagen, Germantown, MD, USA) and run on a PyroMark 96 pyrosequencing machine. As depicted in eFigure 1, the methylation values attained with the 450 K array and the bisulfite sequencing data were highly correlated ($r = 0.92$, $p < .001$; $\rho = 0.88$, $p < .001$).

Cell heterogeneity

Since methylation values at CpG sites can be cell-type specific [19], to control the cell heterogeneity, we estimated the relative proportion of each cell type (e.g., CD34, CD14, and buccal cells) in our heterogenous peripheral saliva samples using a modified version of Housemann et al.'s cell composition estimation analysis, as detailed in prior reports [23, 24].

Race/ethnicity effects

DNA methylation can vary by race or ethnicity [25, 26]; however, little is known about how large the effect of population stratification can be in the methylation studies. To adjust for possible population stratification within the predominantly Caucasian sample, we utilized a methylation-based principal component (PC) approach based on the sets of CpG sites within 50 kb of SNPs, using the 1000 Genomes Project variants with minor allele frequency (MAF) > 0.1 following the procedures developed by Barfield et al. [27].

Resting-state functional connectivity data

The imaging data were acquired at the University of Vermont on a Philips Achieva 3 Tesla scanner in the axial oblique plane with Anterior to Posterior phase encoding using a 2D gradient echo EPI sequence (TR = 2000 ms, TE = 35 ms, flip angle = 90°, matrix = 64×64, FOV = 240×240 mm^2, slice thickness = 4 mm, slice gap of 10%). The VTA and mPFC were selected as seeds in these exploratory analyses, given their role in reward circuitry and the known expression of *OTX2* in these brain regions. Resting-state functional magnetic resonance imaging (rsfMRI) was acquired on 67 children. Of this, 47 participants with (1) acceptable levels of motion during rsfMRI acquisition (17 were excluded for this reason; see online methods for detail on movement-based exclusion), (2) *OTX2* methylation data, and (3) MFQ scores were included in the analyses. The subset of children with imaging data were comparable to the remainder of the sample in terms of age and sex distribution, proportion of youth with clinically significant depressive symptomatology, and range of Y-VACS scores (p = ns, all comparisons). The methods used to analyze the imaging data for these exploratory analyses are detailed in the Supplementary Information online.

Data analyses

Given the large number of CpG sites included on the 450 K beadchip in *OTX2*, the genes regulated by *OTX2* and the negative control genes examined, to build the models for subsequent analyses zero-order correlations were first examined between children's depression scores and the methylation values at these

CpG sites. Table 2 depicts the number of CpG sites examined in *OTX2* and each of the genes regulated by *OTX2*, the number of sites per gene that correlated significantly with children's depression scores at nominal levels of significance ($p < .05$), the number that correlated after Bonferroni correction, the value of the highest correlation among the CpG sites tested in each gene, the Illumina ID associated with that CpG site, and the location of that CpG site which was then used in the analyses reported in this manuscript.

Given the heteroscedasticity of beta values, as recommended by Du et al. [28], *M*-values (logit transformation in the log2 scale) were used in all analyses. In order to take familial correlations into consideration while examining methylation biomarkers and childhood adversity predictors of depression, the data were analyzed using Generalized Estimating Equations (GEE). Demographic factors (e.g., age, sex, and race), cell heterogeneity measures (e.g., CD34, CD14, and buccal cell proportions), and top three PCs were also examined as covariates in the analyses, including the neuroimaging analyses. When examining the impact of *OTX2*-regulated genes, the effect of *OTX2* gene methylation was also included in the models. Analyses were then conducted to determine if the effects of intrafamilial trauma on depression scores are mediated through methylation levels of *OTX2* and related genes, with these analyses considered secondary due to the cross-sectional nature of the data set and the absence of published or publicly available databases to estimate the association of saliva and brain tissue *OTX2* methylation or the role of specific CpG methylation sites on gene expression.

RESULTS

OTX2 methylation-depression analyses

As depicted in Table 2, of the 31 CpG sites located in *OTX2* on the 450 K chip, methylation values in 12 (39%) correlated significantly with children's depression scores at nominal levels of significance and two (6%) correlated significantly after Bonferroni correction for multiple comparisons ($p < .0016$). The two sites that survived the correction for multiple comparisons were located in the 5′UTR region: Illumina ID cg19976235 ($r = 0.28$, $p < .001$) and Illumina ID cg23706497 ($r = 0.33$, $p < .0001$). Methylation values at cg23706497 correlated significantly with the methylation values at cg19976235 ($r = 0.63$, $p < .001$) and with methylation at 25 of the other 30 CpG sites (83%) included in the array. Correlations ranged from −0.64 to 0.73, with negative correlations observed between methylation values at cg23706497 and methylation values at CpG sites on the gene body, and positive correlations observed between methylation values at cg23706497 and methylation values at CpG sites on TSS and other 5′UTR-associated sites.

As depicted in Table 3, methylation in *OTX2* at cg23706497 was a significant predictor of depression in children after accounting for children's intrafamilial adversity scores and controlling for all the relevant covariates. Greater depression scores were associated with greater methylation at this site. As depicted in eFigure 2, there was modest support for a mediation model, with the effects of intrafamilial childhood adversities on children's depression scores showing a trend in the mediation model and the main effects of trauma and *OTX2*, both significant predictors.

Correlations between OTX2 CpG site methylation and CpG sites examined in genes regulated by OTX2

Methylation at *OTX2* cg23706497 correlated significantly with methylation at the sites examined in *RBP3* ($r = -0.24$, $p < .002$), *SEMA3C* ($r = -0.28$, $p < .001$), *THBS4* ($r = -.31$, $p < .001$), *WNT1* ($r = .38$, $p < .001$), and *PAX2* ($r = -0.39$, $p < .001$), and showed a trend in correlating with the site examined in *COL8A1* ($r = -0.13$, $p = .10$).

OTX2-regulated genes-depression analyses

The results of the analyses that examined the impact of adverse childhood experiences, the contribution of *OTX2* gene methylation, and methylation in the genes regulated by *OTX2* are depicted in Table 4. The genes regulated by OTX2 were each significant predictors of depression in children after accounting for the effects of intrafamilial trauma and the effect of *OTX2* methylation. The effect of methylation in the *OTX2*-regulated genes were significant for *PAX6* and *WNT1* with all the covariates included in the analyses, and the effect of methylation at sites examined in the other genes were only significant after using more simplified models and removing the non-significant covariates from the analyses. As depicted in eFigure 3 and eFigure 4, in the models examining *OTX2* together with *PAX6 and WNTI1*, there was also modest support for a mediation model with effects of intrafamilial childhood adversities on children's depression scores showing a trend in the mediation model and the main effects of trauma, *OTX2, PAX6*, and *WNT1* significant in the models.

Re-analyses using targeted bisulfite sequencing data

As indicated above, only the CpG sites for *OTX2, PAX6, WNT1* genes used in primary analyses were validated with bisulfite sequencing. In re-running the analyses with the targeted bisulfite sequencing data for these three sites, the methylation values were first converted to *M*-values. After accounting for the effects of intrafamilial trauma and covariates, both *PAX6* (Wald statistic = 5.72, df = 1, $p < .02$) and *WNT1* (Wald statistic = 4.76, df = 1, $p < .03$) were significant predictors of depression, and the effect of *OTX2* showed a trend toward significance (Wald statistic = 3.07, df = 1, $p < .08$). The less robust findings pertaining to *OTX2* may be a function of the slightly reduced sample size in the sequencing data ($N = 147$); however, the pyrosequencing data and the array results are largely consistent.

Negative control gene analyses

As depicted in eTable 2 and eTable 3 online, while each of the negative control genes had some CpG sites that had significant zero-order correlations with child depression measure, none were significant predictors of depression when child maltreatment and relevant covariates were included in the GEE models, with Wald Statistic values ranging from 0.001 ($p = 0.97$) to 1.23 ($p = 0.27$). The effects of negative control genes remained non-significant even when reduced models were used that only included the significant covariates.

Resting-state functional connectivity data

Using familywise error (FEW) cluster correction with initial selection threshold set to $p < 0.01$, no connectivity findings from the VTA seed retained significance. As depicted in Fig. 1, after controlling for relevant covariates and FEW correction, *OTX2* methylation and MFQ depression scores were predictive of functional connectivity between the right vmPFC and bilateral regions of the medial frontal cortex and the cingulate. The global conjunction illustrates that connectivity between the right vmPFC seed and target regions increased with increased methylation of *OTX2* (Fig. 1b), while connectivity decreased with greater symptoms of depression (MFQ, Fig. 1c). Two clusters of activation reached significance: a cluster centered within the subcallosal gyrus of the cingulate and extended into the ventral frontal medial cortex; a second cluster centered within the ventral aspect of the frontal pole, extending dorsally and posterior to the paracingulate gyrus. No additional seeds reached significance for the targeted conjunction.

DISCUSSION

The data presented in this manuscript suggest a potential role for *OTX2* in conferring risk for depression in children following early

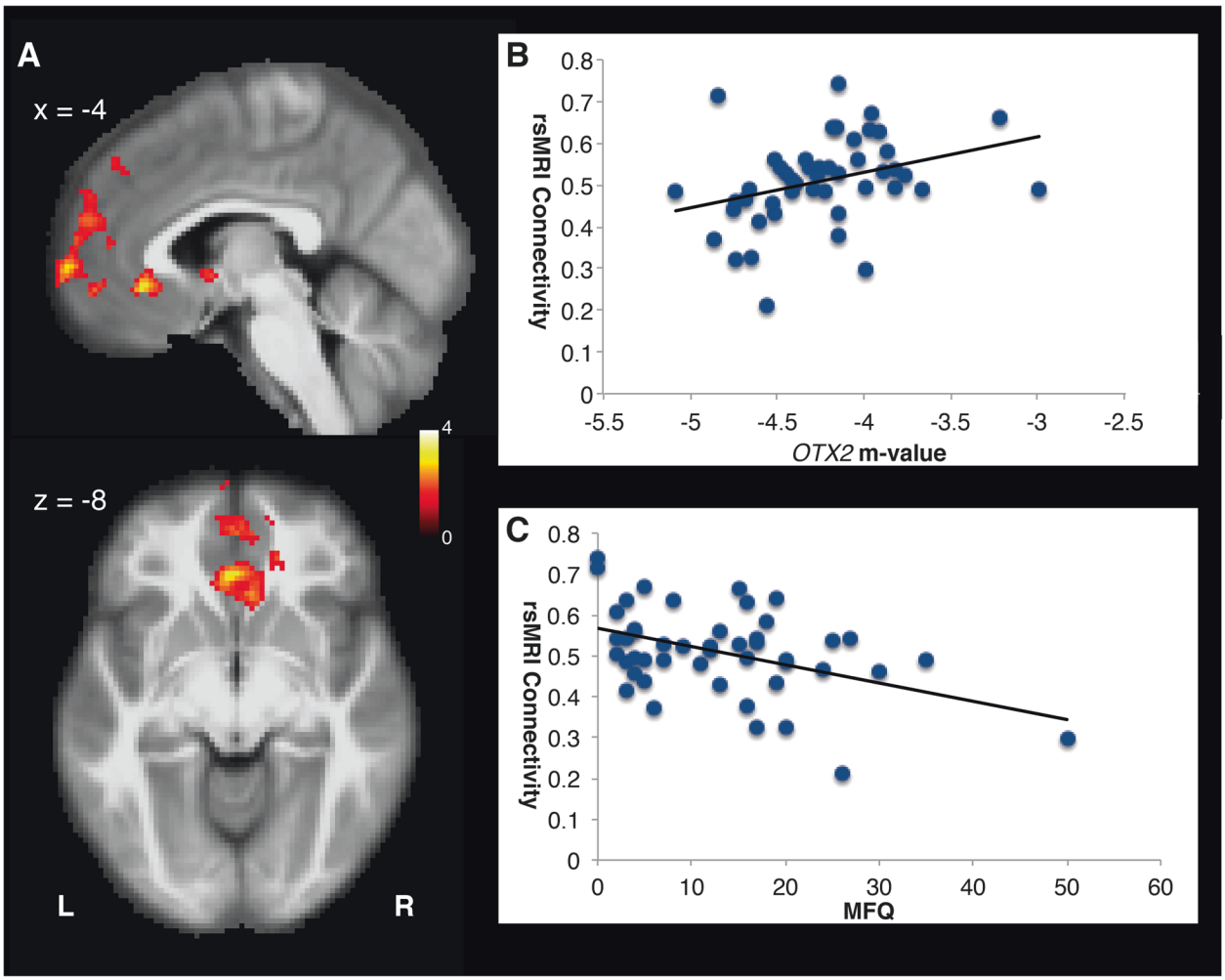

Fig. 1 OTX2 gene methylation and functional connectivity from ventral mPFC. **a** Results of connectivity conjunction analysis showing functional connectivity between the right vmPFC and bilateral regions of the medial frontal cortex and the cingulate. Greater *OTX2* methylation (**b**) and lower depression scores (**c**) are associated with increased connectivity between these two brain regions. Two clusters of activation reached significance: a cluster centered within the subcallosal gyrus of the cingulate and extended into the ventral frontal medial cortex; a second cluster centered within the ventral aspect of the frontal pole, extending dorsally and posterior to the paracingulate gyrus

Table 2. Descriptive information about genes and CpG sites examined

Gene	# CpG sites in gene	# Sites correlate w depression ($p < .05$)	# Sites correlate w depression w bonferroni correction	Highest correlation w depression	Illumina ID of site	Site location
OTX2	31	12 (39%)	2 (6%)	0.33	cg23706497	5′UTR
COL8A1	16	8 (50%)	7 (44%)	−0.31	cg18344769	Body
PAX6	105	21 (20%)	3 (3%)	0.36	cg04938549	body
RBP3	15	5 (33%)	1 (7%)	−0.35	cg07304173	1st Exon
SEMA3C	20	11 (55%)	8 (40%)	−0.38	cg08230910	body
THBS4	18	4 (22%)	3 (17%)	−0.34	cg22636631	body
WNT1	23	6 (26%)	—	−0.19	cg27196808	body

The table above depicts the number of CpG sites examined in *OTX2* and each of the genes regulated by *OTX2*, the number of sites per gene that correlated significantly with children's depression scores at nominal levels of significance ($p < .05$) and after Bonferroni correction (BC), the value of the highest correlation among the CpG sites tested in each gene, the Illumina ID associated with that CpG site which was then used in the follow-up analyses, and the location of that CpG site. Bonferoni correction values were determined by dividing .05 by the number of CpG sites examined in that gene
BC Bonferroni correction, *OTX2* orthodenticle homeobox 2, *COL8A1* collagen type VIII alpha 1 chain, *PAX6* paired box six, *RBP3* retinol-binding protein 3, *SEMA3C* semaphorin-3C, *THBS4* thrombospondin-4, *WNT1* WNT family member 1

adversity, and support the translational applicability of the recently reported mouse model of early life stress [1]. In the current investigation, measures of intrafamilial adversity (e.g., physical abuse, sexual abuse, neglect, witnessing domestic violence) and peripheral saliva-derived DNA measures of *OTX2* methylation were associated with individual differences in depression in children after controlling for demographic factors, cell heterogeneity, and three principal components to prevent spurious findings associated with population stratification effects.

In the current investigation, *OTX2* gene methylation in the periphery was also associated with methylation in the targeted genes downregulated by *Otx2* in the mouse model of early life stress (*Col8a1, Rbp3, Sema3c, Thbs4, Pax6,* and *Wnt1*) [1]. In addition, CpG site methylation in each of the genes regulated by *OTX2* were also shown to be significant predictors of depression in the children after accounting for the effects of intrafamilial trauma and the effect of *OTX2* methylation, with the strongest findings seen for *PAX6* and *WNT1*. *PAX6* acts as a master control gene for neurogenesis and a positive regulator for chromatin remodeling-mediated gene expression [29]. *PAX6* is also critical for differentiation of dopaminergic neurons arising from neural stem cells residing in the dorsal paraventricular zone [30]. *WNT1* plays a role in the establishment of midbrain-dopaminergic neurons [31] and is a major regulator of neural plasticity in the amygdala and hippocampus following acute stress and fear conditioning [2]. Given the role of *WNT1* in neuroplastic processes, it has also been proposed as a "master regulator", which may serve as an important therapeutic target in the treatment of stress-related psychiatric disorders [2]. None of the negative control genes

Table 3. *OTX2* methylation and intrafamilial adversity predictors of depression in children ($N = 157$)

Source	Wald χ^2	df	*p*-value
Age	0.49	1	ns
Sex	1.16	1	ns
Race	3.19	2	ns
CD34	1.77	1	ns
CD14	2.76	1	ns
Buccal	0.21	1	ns
PC1	6.05	1	.014
PC2	5.72	1	.017
PC3	2.65	1	.103
Intrafamilial adversity	5.23	1	.022
OTX2	7.37	1	.007

Methylation in *OTX2* (cg23706497) was significantly associated with depression scores in children after accounting for the effects of experiences of intrafamilial adversity and controlling for relevant covariates
PC principal components, *OTX2* orthodenticle homeobox 2

Table 4. Methylation in *OTX2*-regulated genes, *OTX2* methylation, and intrafamilial adversity predictors of depression in children ($N = 157$)

PAX6 GEE analysis				*WNT1* GEE analysis			
Source	Wald χ^2	df	*p*-value	Source	Wald χ^2	df	*p*-value
Age	0.60	1	ns	Age	0.44	1	ns
Sex	1.23	1	ns	Sex	1.19	1	ns
Race	3.29	2	ns	Race	1.12	2	ns
CD34	2.01	1	ns	CD34	1.19	1	ns
CD14	2.26	1	ns	CD14	3.12	1	.077
Buccal	2.14	1	ns	Buccal	.088	1	ns
PC1	7.91	1	.005	PC1	12.47	1	.001
PC2	7.79	1	.005	PC2	8.95	1	.003
PC3	0.45	1	ns	PC3	2.00	1	ns
Intrafamilial adversity	3.99	1	.046	Intrafamilial adversity	4.38	1	.036
OTX2	7.29	1	.007	*OTX2*	6.46	1	.011
PAX6	4.07	1	.044	*WNT1*	4.84	1	.028
***COL8A* GEE analysis**				***RBP3* GEE analysis**			
PC1	6.20	1	.013	PC1	4.82	1	.028
PC2	5.99	1	.014	PC2	4.92	1	.027
Intrafamilial adversity	3.56	1	.06	Intrafamilial adversity	5.06	1	.024
OTX2	8.29	1	.004	*OTX2*	7.11	1	.008
COL8A1	4.21	1	.04	*RBP3*	6.25	1	.012
***SEMA3C* GEE analysis**				***THSB4* GEE analysis**			
PC1	5.39	1	.02	PC1	5.50	1	.018
PC2	5.30	1	.02	PC2	5.50	1	.019
Intrafamilial adversity	3.65	1	.056	Intrafamilial adversity	4.32	1	.038
OTX2	8.37	1	.004	*OTX2*	8.44	1	.004
SEMA3C	4.58	1	.032	*WNT1*	4.32	1	.038

Methylation in *OTX2*-related genes were significantly associated with depression scores in children after accounting for the effect of experiences of intrafamilial adversity, *OTX2* gene methylation, and controlling for relevant covariates
PC principal components, *OTX2* orthodenticle homeobox 2, *PAX6* paired box six, *WNT1* WNT family member 1, *COL8A1* collagen type VIII alpha 1 chain, *RBP3* retinol-binding protein 3, *SEMA3C* semaphorin-3C, *THBS4* thrombospondin-4

examined were significant predictors of depression in the GEE models.

In the subset of children with neuroimaging data, after controlling for relevant covariates and FEW correction, results of connectivity conjunction analysis found that increased *OTX2* methylation was associated with increased functional connectivity between the right vmPFC and bilateral regions of the medial frontal cortex and cingulate. Two clusters of activation reached significance: a cluster centered within the subcallosal gyrus of the cingulate and extending into the ventral frontal medial cortex; and a second cluster centered within the ventral aspect of the frontal pole, extending dorsally and posterior to the paracingulate gyrus. The subcallosal cingulate is a key structure implicated in depression, with the subcallosal cingulate part of the connectome of converging white matter bundles affecting mood and behavior, including the forceps minor, uncinate fasciculus, cingulum, and fronto-striatal fibers, and also serving as a site for therapeutic deep brain stimulation [32–35]. The frontal pole and paracingulate gyrus are key structures involved in cognitive control in affective contexts, with activation in these regions decreasing after effective psychotherapeutic depression treatment in response to cognitive control stimuli presented within a sad context [36].

Limitations of the current investigation include: small size of the subsample that completed the imaging protocol, the absence of gene expression data, the cross-sectional nature of the study which limits inferences that can be made regarding the causal consequences of the methylation findings, and the absence of a cohort of depressed children without histories of intrafamilial trauma to determine if *OTX2* is uniquely a biomarker for depression in children with a history of early adversity.

Mouse models of early life stress hold significant promise in helping to unravel the mechanisms by which early adversity confers risk for psychopathology. The utility of novel markers identified in model systems through unbiased genomics, transcriptomics, and proteomics can then be examined as potential biomarkers in carefully characterized clinical samples [37]. The results of the current study suggest a potential role of *OTX2* and *OTX2*-regulated genes in the pathophysiology of stress-related disorders that warrants ongoing systematic evaluation using preclinical, postmortem, and clinical research strategies. Through iterative translational research initiatives, ongoing progress can be made to reduce the burden associated with stress-related psychiatric disorders.

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ADDITIONAL INFORMATION

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REFERENCES

1. Pena CJ, Kronman HG, Walker DM, Cates HM, Bagot RC, Purushothaman I, et al. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science. 2017;356:1185–8. https://doi.org/10.1126/science.aan4491.
2. Maheu ME, Ressler KJ. Developmental pathway genes and neural plasticity underlying emotional learning and stress-related disorders. Learn Mem. 2017;24:492–501. https://doi.org/10.1101/lm.044271.116.
3. Jukic MM, Carrillo-Roa T, Bar M, Becker G, Jovanovic VM, Zega K, et al. Abnormal development of monoaminergic neurons is implicated in mood fluctuations and bipolar disorder. Neuropsychopharmacology. 2015;40:839–48. https://doi.org/10.1038/npp.2014.244.
4. Vernay B, Koch M, Vaccarino F, Briscoe J, Simeone A, Kageyama R, et al. Otx2 regulates subtype specification and neurogenesis in the midbrain. J Neurosci. 2005;25:4856–67.
5. Simeone A, Puelles E, Acampora D. The Otx family. Curr Opin Genet Dev. 2002;12:409–15.
6. Puelles E, Annino A, Tuorto F, Usiello A, Acampora D, Czerny T, et al. Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. Development. 2004;131:2037–48.
7. Sugiyama S, Di Nardo AA, Aizawa S, Matsuo I, Volovitch M, Prochiantz A, et al. Experience-dependent transfer of Otx2 homeoprotein into the visual cortex activates postnatal plasticity. Cell. 2008;134:508–20. https://doi.org/10.1016/j.cell.2008.05.054.
8. Lee HHC, Bernard C, Ye Z, Acampora D, Simeone A, Prochiantz A, et al. Genetic Otx2 mis-localization delays critical period plasticity across brain regions. Mol Psychiatry. 2017;22:680–8. https://doi.org/10.1038/mp.2017.1.
9. Beurdeley M, Spatazza J, Lee HH, Sugiyama S, Bernard C, Di Nardo AA, et al. Otx2 binding to perineuronal nets persistently regulates plasticity in the mature visual cortex. J Neurosci. 2012;32:9429–37. https://doi.org/10.1523/JNEUROSCI.0394-12.2012.
10. Spatazza J, Lee HH, Di Nardo AA, Tibaldi L, Joliot A, Hensch TK, et al. Choroid-plexus-derived Otx2 homeoprotein constrains adult cortical plasticity. Cell Rep. 2013;3:1815–23. https://doi.org/10.1016/j.celrep.2013.05.014. Epub 2013 Jun 1813.
11. Kaufman J. Depressive disorders in maltreated children. J Am Acad Child Adolesc Psychiatry. 1991;30:257–65.
12. Kaufman J, Cicchetti D. The effects of maltreatment on school-aged children's socio-emotional development: assessments in a day camp setting. Dev Psychol. 1989;25:516–24.
13. Kaufman J, Yang BZ, Douglas-Palumberi H, Houshyar S, Lipschitz D, Krystal J, et al. Social supports and serotonin transporter gene moderate depression in maltreated children. Proc Natl Acad Sci U S A. 2004;101:17316–21.
14. Bernstein D. A new screening measure for detecting 'Hidden' domestic violence. Psychiatr Times. 1998;15:448–53.
15. Pynoos, RS, Rodriguez, N, Steinberg, A. PTSD index for DSM-5. Los Angeles, CA: University of California Los Angeles; 2013.
16. Kaufman J, Jones B, Steiglitz E, Vitulano L, Mannarino A. The Use of Multiple Informants to Assess Children's Maltreatment Experiences. Journal of Family Violence. 1994;9:227–48.
17. Holbrook, H, et al. The Yale-Vermont Adversity in Childhood Scale: A Quantitative Approach to Adversity Assessment. In: American Academy of Child and Adolescent Psychiatry's 61st Annual Meeting. San Diego, CA; 2015.
18. Angold A, Costello EJ, Messer S, Pickles A, Winder F, Silver D. Development of a Short Questionnaire for use in Epidemiological Studies of Depression in Children and Adolescents. International Journal of Methods in Psychiatric Research. 1995;5:237–49.
19. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, A.P. F, Hansen KD, Irizarry RA. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA Methylation microarrays. . Bioinformatics. 2014;30:363–1369.
20. Wood AR, Esko T, Yang J, Vedantam S, Pers TH, Gustafsson S, et al. Defining the role of common variation in the genomic and biological architecture of adult human height. Nat Genet. 2014;46:1173–86. https://doi.org/10.1038/ng.3097.
21. Danese A, Tan M. Childhood maltreatment and obesity: systematic review and meta-analysis. Mol Psychiatry. 2014;19:544–54.
22. Yang B-Z, Zhang H, Ge W, Weder N, Douglas-Palumberi H, Perepletchikova F, et al. Child Abuse and Epigenetic Mechanisms of Disease Risk. American Journal of Preventive Medicine. 2013;44:101–7.
23. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86. https://doi.org/10.1186/1471-2105-13-86.
24. Smith AK, Kilaru V, Klengel T, Mercer KB, Bradley B, Conneely KN, et al. DNA extracted from saliva for methylation studies of psychiatric traits: evidence tissue specificity and relatedness to brain. Am J Med Genet B Neuropsychiatr Genet. 2015;168B:36-44. https://doi.org/10.1002/ajmg.b.32278.
25. Adkins RM, Krushkal J, Tylavsky FA, Thomas F. Racial differences in gene-specific DNA methylation levels are present at birth. Birth Defects Res A Clin Mol Teratol. 2011;91:728–36. https://doi.org/10.1002/bdra.20770.

26. Heyn H, Moran S, Hernando-Herraez I, Sayols S, Gomez A, Sandoval J, et al. DNA methylation contributes to natural human variation. Genome Res. 2013;23:1363–72. https://doi.org/10.1101/gr.154187.112.
27. Barfield RT, Almli LM, Kilaru V, Smith AK, Mercer KB, Duncan R, et al. Accounting for population stratification in DNA methylation studies. Genet Epidemiol. 2014;38:231–41. https://doi.org/10.1002/gepi.21789.
28. Du P, Zhang X, Huang C, Jafari N, Kibbe W, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.
29. Soumiya H, Fukumitsu H, Furukawa S. Prenatal immune challenge compromises the normal course of neurogenesis during development of the mouse cerebral cortex. J Neurosci Res. 2011;89:1575–85. https://doi.org/10.1002/jnr.22704.
30. De Chevigny A, Core N, Follert P, Wild S, Bosio A, Yoshikawa K, et al. Dynamic expression of the pro-dopaminergic transcription factors Pax6 and Dlx2 during postnatal olfactory bulb neurogenesis. Front Cell Neurosci. 2012;6:6. https://doi.org/10.3389/fncel.2012.00006.
31. Prakash N, Brodski C, Naserke T, Puelles E, Gogoi R, Hall A, et al. A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. Development. 2006;133:89-98.
32. Riva-Posse P, Choi KS, Holtzheimer PE, Crowell AL, Garlow SJ, Rajendra JK, et al. A connectomic approach for subcallosal cingulate deep brain stimulation surgery: prospective targeting in treatment-resistant depression. Mol Psychiatry. 2017;11:59.
33. Akil H, Gordon J, Hen R, Javitch J, Mayberg H, McEwen B, et al. Treatment resistant depression: A multi-scale, systems biology approach. Neurosci Biobehav Rev. 2018;84:272–88.
34. Mayberg HS, Brannan SK, Mahurin RK, Jerabek PA, Brickman JS, Tekell JL, et al. Cingulate function in depression: a potential predictor of treatment response [see comments]. Neuroreport. 1997;8:1057–61.
35. Ducharme S, Albaugh MD, Hudziak JJ, Botteron KN, Nguyen TV, Truong C, et al. Anxious/Depressed Symptoms are Linked to Right Ventromedial Prefrontal Cortical Thickness Maturation in Healthy Children and Young Adults. Cereb Cortex. 2014;24:2941–50. Epub 2013 Jun 2907 https://doi.org/10.1093/cercor/bht151.
36. Dichter GS, Felder JN, Smoski MJ. The effects of brief behavioral activation therapy for depression on cognitive control in affective contexts: an fMRI investigation. J Affect Disord. 2010;126:236–44. https://doi.org/10.1016/j.jad.2010.03.022.
37. Kaufman, J., Unraveling the Genetics of Major Depression and Stress-Related Psychiatric Disorders: Is it Time for a Paradigm Shift? Biological Psychiatry, 2018. 84(2): p. 82–4