Beyond genotype: serotonin transporter epigenetic modification predicts human brain function

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We examined epigenetic regulation in regards to behaviorally and clinically relevant human brain function. Specifically, we found that increased promoter methylation of the serotonin transporter gene predicted increased threat-related amygdala reactivity and decreased mRNA expression in postmortem amygdala tissue. These patterns were independent of functional genetic variation in the same region. Furthermore, the association with amygdala reactivity was replicated in a second cohort and was robust to both sampling methods and age.

The systematic integration of human molecular genetics and in vivo neuroimaging have contributed to our increasing understanding of how DNA sequence–based genetic variation shapes individual differences in brain function, complex behavioral traits and related risk for psychopathology. Parallel research in animal models has highlighted a critical role for non-sequence–based epigenetic variation in the emergence of individual differences in brain function and risk-related behavior. The importance of similar epigenetic mechanisms for behaviorally and clinically relevant brain function in humans has yet to be fully explored.

We used bisulfite sequencing to determine the percentage of methylation of the proximal promoter region of the serotonin transporter gene (SLC6A4) in saliva-derived DNA from a discovery cohort of 80 young adults and blood-derived DNA from an independent replication cohort of 96 adolescents. We targeted SLC6A4 because it encodes the serotonin transporter, which modulates brain function and behavior by regulating the duration and intensity of synaptic serotonin signaling. Dysfunction of the serotonin transporter has also been implicated in the pathophysiology of mood and anxiety disorders, and pharmacologic blockade of this molecule is the primary mode of treating these same disorders.

We focused our analyses on the 20 CpG sites closest to the transcription start site (TSS) of SLC6A4 exhibiting substantial variability across individuals (Table 1 and Supplementary Fig. 1a,b). Additional proximal promoter sites were excluded, as we found virtually no variability across individuals (Supplementary Table 1).

In light of recent work suggesting that methylation immediately downstream of the TSS may also affect transcription, we sampled additional CpG sites up to 119 base pairs downstream of the TSS, spanning exon 1 and intron 1 (Supplementary Table 1), whose effects were investigated in separate control analyses.

We evaluated the relationship between SLC6A4 proximal promoter methylation and threat-related amygdala reactivity assayed using blood oxygen level–dependent (BOLD) functional magnetic resonance imaging (fMRI). We selected this neural phenotype as a measure of behaviorally and clinically relevant brain function because it is clearly involved in the emergence of both normal and pathologic emotional behaviors. Notably, these behaviors include responsivity to environmental and social stress, which is associated not only with epigenetic modification, but also with variability in serotonin signaling. Moreover, there is now ample evidence linking variability in serotonin signaling with individual differences in amygdala reactivity.

In our discovery cohort, the percentage of methylation of the SLC6A4 proximal promoter was positively correlated with threat-related amygdala reactivity in the left hemisphere (adjusted $R^2 = 0.067, b = 0.282, P = 0.011$; Fig. 1a,b). This effect was observed at a trend level in the right hemisphere (adjusted $R^2 = 0.032, b = 0.211, P = 0.060$). The percentage of methylation continued to account for significant variability in left amygdala reactivity even when controlling for possible effects of gender, age, early and recent life stress, and current psychiatric disorder (left hemisphere: $R^2 = 0.084, b = 0.292, P = 0.009$; right hemisphere: $R^2 = 0.045, b = 0.214, P = 0.060$).

Similar results were obtained when using the top principal component (PC) capturing 24% of the methylation variance in the same region (Supplementary Tables 2 and 3, and Supplementary Fig. 2a,b). In an exploratory follow-up analysis, we probed the effects of individual CpG site methylation levels on these same phenotypes and found that CpG 14 (188 base pairs upstream of TSS) showed the strongest association effects across both hemispheres (Table 1).

Given prior work establishing predictive links between genetic variation and amygdala reactivity, we next compared the effect of SLC6A4 proximal promoter methylation on amygdala reactivity to that of the serotonin transporter–linked polymorphic region (5-HTTLPR) and rs25531, which together define a functional tri-allelic polymorphism previously associated with variability in amygdala reactivity and responsiveness to stress. SLC6A4 methylation continued to predict amygdala reactivity even when the 5-HTTLPR/rs25531 genotype was accounted for alongside all other covariates (left hemisphere: $R^2 = 0.086, b = 0.296, P = 0.009$; right hemisphere: $R^2 = 0.043, b = 0.211, P = 0.066$). This suggests that

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there is a preponderance of epigenetic variation over sequence-based variation in regulatory regions of the same gene.

As a negative control, we examined the correlation between amygdala reactivity and methylation in other regions of SLC6A4 and the COMT gene, which codes for an enzyme responsible for regulating catecholamine, but not serotonin, signaling. As expected, there were no significant correlations between the two hemispheres 12. In the replication cohort, the single site showing strongest correlations with amygdala reactivity and methylation in other regions of SLC6A4 (P < 0.10). Similarly, there were also no significant correlations between amygdala reactivity and methylation in other regions of SLC6A4.

Table 1 Summary of results from linear regression models predicting in vivo amygdala reactivity and amygdala tissue SLC6A4 mRNA from percentage methylation at each of the 20 individual proximal promoter CpG sites sampled across the discovery, replication and postmortem cohorts

| CpG site | Distance to TSS | Discovery cohort (DNS) | Replication cohort (TAOS) | Postmortem | Amygdala SLC6A4 mRNA |
|----------|----------------|------------------------|---------------------------|------------|---------------------|
|          |                | Left amygdala          | Right amygdala            | Left amygdala | Right amygdala | Amygdala SLC6A4 mRNA |
|          |                | b | p | b | p | b | p | b | p |
| 1        | –69            | 0.163 | 0.149 | 0.180 | 0.11 | 0.112 | 0.278 | –0.061 | 0.556 |
| 2        | –72            | 0.263 | 0.018 | 0.125 | 0.269 | 0.11 | 0.043 | 0.511 | 0.15 |
| 3        | –99            | 0.094 | 0.409 | 0.052 | 0.647 | 0.19 | 0.248 | 0.18 | 0.500 |
| 4        | –112           | 0.224 | 0.046 | 0.279 | 0.012 | 0.107 | 0.297 | 0.130 | 0.208 |
| 5        | –133           | 0.102 | 0.369 | 0.138 | 0.223 | 0.229 | 0.252 | 0.707 | –0.073 |
| 6        | –135           | 0.224 | 0.046 | 0.260 | 0.02 | 0.129 | 0.209 | –0.106 | 0.306 |
| 7        | –139           | 0.069 | 0.544 | 0.191 | 0.089 | 0.164 | 0.109 | 0.019 | 0.856 |
| 8        | –141           | 0.026 | 0.818 | 0.048 | 0.672 | 0.010 | 0.921 | 0.020 | 0.846 |
| 9        | –147           | 0.128 | 0.258 | 0.058 | 0.608 | 0.196 | 0.056 | –0.056 | 0.587 |
| 10       | –149           | 0.280 | 0.012* | 0.225 | 0.045 | 0.214 | 0.037 | 0.133 | 0.195 |
| 11       | –155           | 0.186 | 0.099 | 0.240 | 0.032 | 0.264 | 0.070 | 0.196 | 0.290 |
| 12       | –170           | 0.162 | 0.151 | 0.144 | 0.204 | 0.273 | 0.007 | 0.213 | 0.037 |
| 13       | –174           | 0.245 | 0.028 | 0.219 | 0.051 | 0.305 | 0.003* | 0.219 | 0.032* |
| 14       | –188           | –0.029 | 0.799 | 0.287 | 0.010* | 0.218 | 0.033 | 0.097 | 0.345 |
| 15       | –190           | 0.157 | 0.164 | 0.164 | 0.146 | 0.226 | 0.027 | 0.037 | 0.722 |
| 16       | –195           | 0.094 | 0.407 | –0.103 | 0.363 | 0.082 | 0.424 | 0.004 | 0.965 |
| 17       | –200           | 0.006 | 0.955 | –0.107 | 0.343 | 0.226 | 0.027 | 0.014 | 0.893 |
| 18       | –207           | 0.148 | 0.189 | 0.091 | 0.423 | 0.235 | 0.021 | 0.029 | 0.782 |
| 19       | –209           | 0.119 | 0.294 | –0.059 | 0.602 | 0.115 | 0.263 | –0.116 | 0.261 |
| 20       | –213           | 0.100 | 0.376 | –0.057 | 0.616 | 0.129 | 0.209 | –0.106 | 0.306 |

Results from the in vivo imaging cohorts are not adjusted for covariates. In light of gender, age, pH, postmortem interval and RNA ratio effects in the postmortem cohort, the results for the postmortem findings are adjusted for covariates. The CpG site numbering scheme reflects the ordering of CpG site in this proximal promoter region and has no relation to any unique CpG site numerical identifiers. All significant P values are shown in bold.

* CpG site with strongest association for each phenotype.

Figure 1 Effects of SLC6A4 proximal promoter methylation on amygdala reactivity. (a,c) Statistical parametric map illustrating mean bilateral threat-related amygdala reactivity across all participants in the discovery cohort (left: x = –24, y = –8, z = –16, t = 10.29, P = 2.00995 × 10–14, k = 180; right: x = 30, y = 4, z = –20, t = 11.13, P < 0.00001, k = 203). (b,d) The average percent SLC6A4 proximal promoter methylation was positively correlated with reactivity of the left amygdala in both the discovery cohort and replication cohort. Adjusted R^2 = 0.067.
pharmacologic fMRI\textsuperscript{14,15} studies linking relatively increased serotonin signaling with increased amygdala reactivity, as well as with observations that methylation in or near promoter regions generally inhibits gene transcription\textsuperscript{4}. In light of these data, and to gain further mechanistic insight into our in vivo findings, we examined the effect of percent methylation of the same 20 CpG sites sampled in our imaging cohorts on serotonin transporter mRNA levels in postmortem amygdala tissue from a third independent cohort of 34 individuals.

As expected, clinical and biochemical parameters (that is, diagnostic status, pH and RNA ratio) influenced mRNA levels ($P < 0.091$). Thus, these were included as covariates alongside age, gender and postmortem interval in all analyses involving postmortem data. When controlling for the effects of these parameters, there was no significant association between overall percent methylation and mRNA in amygdala tissue ($P = 0.699$). However, a site-specific investigation revealed a significant negative correlation ($P = 0.039$) between mRNA levels and percent methylation at CpG 14, which exhibited the strongest correlation with amygdala reactivity in both our imaging cohorts (Table 1 and Fig. 2). As with our in vivo imaging data, this epigenetic effect was further independent of 5-HTTLPR/rs25531 genotype ($P = 0.031$).

Collectively, our results suggest that methylation of the proximal promoter of human SL\textit{C6A4} predicts threat-related amygdala reactivity, possibly reflecting decreased serotonin transporter gene expression and, consequently, reduced regional serotonin reuptake. Moreover, these epigenetic effects are independent of, and greater than, the effects of the 5-HTTLPR/rs25531 functional polymorphism near the same genomic region. Further demonstrating the independence of these genetic and epigenetic effects, none of the 20 proximal promoter CpG sites surveyed across the three cohorts overlapped the 5-HTTLPR, which is located 1,400 base pairs upstream of the TSS and primarily affects the distal promoter. In addition, the 5-HTTLPR/rs25531 genotype had no effect on proximal promoter methylation in any of the cohorts ($P > 0.30$).

Although we did not directly map methylation in peripheral tissues onto methylation levels in brain in the same cohort, cross-cohort convergence among tissues (saliva, blood, brain) is consistent with recent work demonstrating a substantial correlation between the blood and brain methylation\textsuperscript{16}. Furthermore, prior work has mapped variability in a similar range of SL\textit{C6A4} promoter methylation values onto individual differences in serotonin biosynthesis in vivo\textsuperscript{17}. Finally, the cross-tissue convergence that we observed suggests that two distinct types of readily assayed peripheral tissues (blood and saliva) could potentially be used as equally valid proxies of neural tissue. A notable limitation of our work is that, as a result of practical constraints, the postmortem tissue analysis was limited to the amygdala, although more informative differences in SL\textit{C6A4} transcript levels are likely to emerge in the dorsal raphe nucleus, where the serotonin transporter is more densely expressed. This limitation notwithstanding, our results demonstrate that meaningful links between the human epigenome and brain can be mapped using DNA derived from readily assayed peripheral tissues. In addition to encouraging careful consideration of the effects of promoter methylation in SL\textit{C6A4} on behaviorally and clinically relevant brain function, we hope that our results will advance broader research on epigenetic mechanisms in the emergence of individual differences in human behavior and related risk for psychopathology.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.S.N. designed the study, participated in the collection of the imaging and genetic data for the discovery cohort, conducted all of the statistical analyses, and wrote the manuscript with A.R.H. K.C.K. and S.G. designed and coordinated the methylation analyses in the discovery cohort. C.-M.W. designed the parent protocol, supervised quantitative PCR experiments and coordinated methylation analyses in the postmortem cohort. E.S. designed the parent protocol and performed the methylation assays in the replication and postmortem cohorts. M.L.S. conducted the quantitative PCR in the postmortem cohort. E.S. designed the parent protocol, performed the quantitative PCR experiments and coordinated methylation analyses in the postmortem cohort. D.E.W. designed the parent protocol for the replication cohort and coordinated the methylation assays in both the replication and postmortem cohorts. A.R.H. designed the study, coordinated all analyses and wrote the manuscript with Y.S.N. He also designed the parent protocol for the discovery cohort and the neuroimaging protocol for the replication cohort. All of the authors provided feedback on the manuscript and approved its final version.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Participants in the discovery cohort were selected for inclusion in analyses involving DNA methylation assays. The DNS assesses a range of behavioral and biological traits among young adult, student volunteers. All participants provided informed consent in accord with Duke University guidelines, and were in good general health. Two participants’ samples displayed incorrect sequence patterns for our SLC6A4 promoter assays as a result of unknown mutations or equipment dispensation error and were excluded from this analysis. Nine additional participants were excluded because of task non-compliance or response box failure, leaving a final sample of 80 individuals (42 women, mean age = 19.74 ± 1.33 years).

All participants were free of the following study exclusions: (1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms, (2) use of psychoactive, glucocorticoid or hypolipidemic medication, and (3) conditions affecting cerebral blood flow and metabolism (for example, hypertension). Diagnosis of any current DSM-IV Axis I disorder or select Axis II disorders (Antisocial Personality Disorder and Borderline Personality Disorder), assessed with the electronic Mini International Neuropsychiatric Interview18 and Structured Clinical Interview for the DSM-IV (SCID) subtests19, respectively, was not an exclusion as the DNS seeks to establish broad variability in multiple behavioral phenotypes related to psychopathology. However, all participants were medication-free at the time of the study. No participants met criteria for either Antisocial or Borderline Personality Disorder, and 16 participants from our final sample (N = 80) met criteria for at least one Axis I disorder. Given that the exclusion of these individuals did not substantially alter our results, we present data from the entire sample in the main text (specific diagnoses available upon request). In addition, all analyses were conducted both with and without current medication for in all analyses involving this sample. A square root transformation was applied to SLC6A4 mRNA levels to normalize its positively skewed and kurtotic distribution (pre-transformation skewness = 1.92, kurtosis = 6.45, post-transformation skewness = 0.65, kurtosis = 1.43).

BOLD fMRI task. As described previously22, the amygdala reactivity procedure used in the discovery cohort (DNS) consisted of four blocks of a face-processing task interleaved with five blocks of a somotor controller task. During task blocks, participants viewed a trio of faces (with neutral, angry, fearful or surprised expressions) and matched 1 of 2 faces (bottom) identical to a target face (top). During control blocks, participants matched simple geometric shapes. In the replication cohort (TAOS), the task consisted only of angry and fearful faces. Thus, for consistency between samples, we focused our analyses on the anger + fear > shapes contrast in our discovery cohort. Performance was monitored and participants with accuracy < 75% were excluded from analysis.

BOLD fMRI data acquisition. Participants in the discovery cohort were scanned using a research-dedicated GE MR750 3T scanner equipped with high-power high-duty cycle 50-mT m⁻¹ gradients at 200 T m⁻¹ s⁻¹ slew rate, and an eight-channel head coil for parallel imaging at high bandwidth up to 1 MHz at the Duke-UNC Brain Imaging and Analysis Center. A semi-automated high-order shimming program was used to ensure global field homogeneity. A series of 34 interleaved axial functional slices aligned with the anterior commissure-posterior commissure (AC-PC) plane were acquired for full-brain coverage using an inverse-spiral pulse sequence to reduce susceptibility artifact (TR = 2,000 ms; TE = 30 ms; flip angle = 60; FOV = 240 mm; 3.75 × 3.75 × 4 mm voxels; interslice skip = 0). Four initial RF excitations were performed (and discarded) to achieve steady-state equilibrium. To allow for spatial registration of each participant’s data to a standard coordinate system, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR = 7,700 ms; TE = 3.0 ms; flip angle = 12; voxel size = 0.9 × 0.9 × 4 mm; FOV = 240 mm; interslice skip = 0).

Participants in the replication cohort were scanned on a Siemens 3T Trio Scanner at the UTHSCSA. BOLD fMRI data were acquired with a gradient-echo echo planar imaging (EPI) sequence (TR = 2,000 ms; TE = 25 ms; flip angle = 70; FOV = 256 mm; 2.00 × 2.00 × 3.00 mm voxels; interslice skip = 0) covering 34 interleaved 3-mm-thick axial slices. As in the discovery cohort, high-resolution three-dimensional structural images were acquired in 34 axial slices co-planar with the functional scans (TR = 5,610 ms; TE = 72 ms; flip angle = 150; voxel size = 0.8 × 0.8 × 3 mm; FOV = 220 mm × 320 mm; interslice skip = 0).

BOLD fMRI data preprocessing. The same data preprocessing steps were applied to both the discovery and the replication cohort. Briefly, images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotaxic space (Montreal Neurological Institute template) using a 12-parameter affine model (final resolution of functional images = 2 mm isotropic voxels), and smoothed to minimize noise and residual difference in gyral anatomy with a Gaussian filter, set at 6-mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean.

Variability in single-subject whole-brain functional volumes was determined using the Artifact Recognition Toolbox (http://www.nitrc.org/projects/artifact_detect). Individual whole-brain BOLD fMRI volumes meeting at least one of two criteria were assigned a lower weight in determination of task-specific effects: significant mean-volume signal intensity variation (that is, within volume mean signal greater or less than 4 s.d. of mean signal of all volumes in time series), and individual volumes where scan-to-scan movement exceeded 2 mm translation or 2° rotation in any direction.

BOLD fMRI data analysis. The general linear model (GLM) of SPM8 (http://www.fil.ion.ucl.ac.uk/spm) was used to conduct fMRI data analyses. Linear contrasts employing canonical hemodynamic response functions were used to estimate differential effects of condition from the contrast of faces > shapes for each individual. Individual contrast images were then used in second-level random effects models accounting for scan-to-scan and participant-to-participant variability to determine mean condition-specific regional responses using one-sample t-tests. Regions of interest masks for the bilateral amygdala were constructed using the automatic anatomical labeling (AAL) in WFU PickAtlas.
Tool, version 1.04. A statistical threshold of \( P < 0.05 \), FWE corrected, and \( \geq 10 \) contiguous voxels was applied to amygdala analyses within each hemisphere. BOLD values from voxels within the amygdala exhibiting strongest main effect of task were extracted using the VOI tool in SPM8. These extracted values were then entered into regression models using IBM SPSS Statistics 20.0 (SPSS). Notably, by extracting amygdala BOLD parameter estimates from the voxels activated by our procedure rather than clusters specifically correlated with our independent variables of interest, we preclude the possibility of any regression coefficient inflation that may result from capitalizing on the same data twice\(^{23}\). We have successfully used this conservative strategy in previous reports\(^{24-26}\).

DNA extraction and 5-HTTLPR/rs25531 genotyping. Saliva samples from discovery cohort participants were collected using Oragene kits and DNA was extracted in accordance with the manufacturer’s guidelines (Oragene, Genotek). In the replication cohort, DNA was extracted from whole blood. Postmortem brain DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen), using a protocol that was modified from manufacturers’ instructions (additional proteinase K and RNase A).

The same 5-HTTLPR/rs25531 genotyping protocol was applied to DNA samples from all three cohorts. Primer sequences for genotyping 5-HTTLPR were described previously\(^{28}\) (forward: 5′- ATGCCAGACCTAACCCTAATGT-3′; reverse: 5′-GAGCCGGCAAGGTTGCCCCGA-3′). PCR was conducted using the following cycling conditions: initial 15-min denaturing step at 95 °C, followed by 35 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 40 s, and a final extension phase of 72 °C for 15 min. Reactions were performed in 10× reaction buffer supplemented with ethidium bromide (0.03%, vol/vol, BDH) and visualized by ultraviolet trans-illumination. Genotype calls were made by three independent raters, who reached consensus on 100% of the discovery and replication cohort samples. Genotype could not be determined accurately for one postmortem sample. Thus, it was removed from analysis, leaving a final sample of 34 individuals (10 women, median age = 49.44 ± 12.02 years).

DNA methylation analyses by quantitative bisulfite pyrosequencing. DNA methylation levels of the proximal promoter of the serotonin transporter gene in the discovery cohort were determined using quantitative bisulfite pyrosequencing by EpigenDx. Briefly, the human serotonin transport gene (SLC6A4) proximal promoter methylation assays analyze 20 CpG dinucleotides in the promoter region from −213 to −69 base pairs of the TSS, based on Ensembl Gene ID ENSG000001108576 and the Transcript ID ENST00000394821. The SLC6A4 promoter assays (AD5880-FS1 and AD5880-FS2) were targeted to the antisense sequence of SLC6A4 gene. The targeted CpG loci are listed in Supplementary Table 1, along with their genomic location and position relative to the transulatory start site. The target sequences (genomic DNA and bisulfite converted DNA) are available upon request.

For each analysis, the bisulfite conversion was performed with 500 ng provided genomic DNA using the EZ DNA methylation kit (ZymoResearch). The PCR reaction was performed based on recommended assay conditions (EpigenDx) using 0.2 μM of each primer with one of the PCR primers being biotinylated in order to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences), and the Sepharose beads containing the immobilized PCR product were purified, washed and denatured using 0.2 M NaOH solution and rewarshed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen) as recommended by the manufacturer. 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer’s instructions (Pyrosequencing, Qiagen). The methylation status of each CpG site was analyzed individually as an artificial T/C single-nucleotide polymorphism using iQ CpG software (Pyrosequencing, Qiagen).

The methylation level at each CpG site was calculated as the percentage of the methylated alleles over the sum of methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites in each targeted region. For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient sodium bisulfite DNA conversion. We also included low, medium and high methylated standards (EpigenDx) as controls in each run. In light of the low methylation values observed at some CpG sites (<4%) in the discovery cohort, additional PCR bias testing was performed by EpigenDx for the entire AD5880 assay (that is, all 20 CpG sites taken together), as well as individually for CpG 14. The bias testing was conducted by mixing the unmethylated DNA control and in vitro methylated DNA at different ratios (0%, 2.5%, 5%, 7.5%, 10%, 50% and 100%) followed by bisulfite modification, PCR and pyrosequencing analysis, which were run in triplicate. There was a high correlation between the percent methylation obtained from the mixing study and expected methylation percentages (\( r^2 > 0.97 \)), which confirms the quality of our data (Supplementary Fig. 1).

In the replication and postmortem cohorts, methylation analysis on the same SLC6A4 proximal promoter 20 CpG sites as targeted by the AD5880FS1 and AD5880FS2 assays was carried out at the Core for Advanced Translational Technologies (uthscsa). The protocol used was the same as in the discovery cohort, except with independently designed PCR and sequencing primers (primer sequences available upon request). Results were analyzed using PyroMark QR6 MD and PyroMark CpG 1.0 software (Qiagen).

Control analyses conducted by EpigenDx only in the discovery cohort used additional methylation assays in exon 1 and intron 1 of SLC6A4 (Supplementary Table 1), as well as the promoter region of the COMT gene (Supplementary Table 4). Detailed assay information is available upon request.

Self-report measures. To assess recent life stress, we administered a modified version of the Life Events Scale for Students (LESS)\(^{29}\). This modified version of the scale asks participants to indicate whether they experienced common stressful life events in the past 12 months; in addition, for each event that occurred, participants reported on the impact it had on their lives on a 1–4 scale (with 1 being the highest). The impact scores were set to zero for events that did not occur. Based on prior research\(^{24,25}\), we focused on the LESS Highest Impact metric, reflecting the highest impact associated with any event which occurred within the past year. We assessed early life trauma using the Childhood Trauma Questionnaire (CTQ)\(^{29}\).

Statistical analysis. Percent methylation was computed as the ratio of methylated cytosines over the sum of all methylation and unmethylated cytosines. Our main analyses focused on two SLC6A4 promoter assays covering a total of 20 CpG sites sampled across all three cohorts (Table 1 and Supplementary Table 1).

No single-nucleotide polymorphisms resulting in CpG site gain or loss were identified in the assayed regions. In addition to analysis using average percent methylation across the 20 CpG sites in our region of interest, we applied principal component analysis (PCA) to these 20 CpG sites in both the discovery and replication cohorts. The unrotated correlation matrix was analyzed to output principal component scores. An eigenvalue greater than 1 indicates that PCs account for more variance than accounted by one of the original variables in standardized data. PCA resulted in five PCs with eigenvalues > 1 in both samples. The first PC exhibited effects similar to those associated with SLC6A4 promoter methylation values averaged across all 20 CpG sites in both the discovery and the replication cohort (Supplementary Tables 2, 3, 5 and 6, and Supplementary Fig. 1). In light of the smaller number of data points (\( n = 34 \) final sample), no PCA was performed in the postmortem cohort, where CpG sites were analyzed individually.
Linear regression models, as implemented in IBM SPSS Statistics 20.0, were used to investigate the linear effect of methylation values (independent variable) on amygdala reactivity or SLC6A4 mRNA levels (dependent variables). Results from two-tailed tests are reported for all analyses.

A Supplementary Methods Checklist is available.

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