Serotonin Transporter Gene Methylation is Associated With Hippocampal Gray Matter Volume

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Abstract: *Background:* The serotonin transporter (5-HTT) and the 5-HTTLPR/rs25531 polymorphisms in its gene (*SLC6A4*) have been associated with depression, increased stress-response, and brain structural alterations such as reduced hippocampal volumes. Recently, epigenetic processes including *SLC6A4* promoter methylation were shown to be affected by stress, trauma, or maltreatment and are regarded to be involved in the etiology of affective disorders. However, neurobiological correlates of *SLC6A4* promoter methylation have never been studied or compared to genotype effects by means of human neuroimaging hitherto. *Methods:* Healthy subjects were recruited in two independent samples ($N = 94$, $N = 95$) to obtain structural gray matter images processed by voxel-based morphometry

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(VBM8), focusing on hippocampal, amygdala, and anterior cingulate gyrus gray matter structure. *SLC6A4* promoter methylation within an *AluJb* element and 5-HTTLPR/rs25531 genotypes were analyzed in view of a possible impact on local gray matter volume. **Results:** Strong associations of *AluJb* methylation and hippocampal gray matter volumes emerged within each sample separately, which in the combined sample withstood most conservative alpha-corrections for the entire brain. The amygdala, insula, and caudate nucleus showed similar associations. The 5-HTTLPR/rs25531 showed no main effect on gray matter, and the effect of methylation rates on hippocampal structure was comparable among the genotype groups. **Conclusions:** Methylation within the *AluJb* appears to have strong effects on hippocampal gray matter volumes, indicating that epigenetic processes can alter brain structures crucially involved in stress-related disorders. Different ways of regulating *SLC6A4* expression might involve exonization or transcription factor binding as potentially underlying mechanisms, which, however, is speculative and warrants further investigation. *Hum Brain Mapp* 35:5356–5367, 2014. © 2014 Wiley Periodicals, Inc.

Key words: epigenetics; DNA methylation; *Alu* element; 5-HTTLPR; MRI; hippocampus

INTRODUCTION

Altered serotonergic neurotransmission appears to play a pivotal role in the etiology of psychiatric disorders such as major depression, one of the most debilitating disorders worldwide [Wong and Licinio, 2001]. The serotonin transporter (5-HTT) is crucial for regulating serotonergic neurotransmission and represents the main target for several antidepressant drugs. Since the discovery of a common functional polymorphism (5-HTTLPR) in the promoter of the 5-HTT gene (*SLC6A4*) [Lesch et al., 1996], a large body of research investigated associations of 5-HTTLPR genotype with depression and depression associated endophenotypes [Caspi and Moffitt, 2006]. Both, 5-HTTLPR and the later discovered functional single nucleotide polymorphism rs25531 have been associated with depression [Clarke et al., 2010], attention bias and limbic hyper-responsiveness [Dannlowski et al., 2007, 2008, 2010; Pergamin-Hight et al., 2012], altered endocrine stress-response [Miller et al., 2013], and reduced hippocampal volumes [Frodl et al., 2008, 2010a].

Although an important portion of gene activity is determined by heritable factors, gene-environment interactions (GxE) might contribute substantially to neuropsychiatric disorders such as depression [McGowan and Kato, 2008; Moffitt et al., 2005], as shown for the interaction of 5-HTTLPR genotype and susceptibility to stressful life events [Karg et al., 2011; Van Ijzendoorn et al., 2012]. However, the variance of transcription accounted for by 5-HTTLPR genotype appears to be moderate at best and epigenetic mechanisms such as DNA methylation might play an even more important regulatory role in gene expression [Jaenisch and Bird, 2003]. This mechanism typically entails that methyl groups are added to cytosine bases at CpG-dinucleotides (CpG sites) [Ptak and Petronis, 2010]. Depending on the position of CpG sites relative to a gene, sites within the promoter or within functional elements like CpG-rich regions (CpG islands) are of particular func-

tional relevance for the expression of this gene and their methylation mostly represses transcription [Moore et al., 2013]. DNA methylation itself is influenced by environmental factors like stress or early-life adversity, and hereby induced methylation changes are often enduring throughout life and are associated with long-term phenotypic changes [Heim and Binder, 2012; Jirtle and Skinner, 2007; Weaver, 2007]. Recent studies indeed reported associations of CpG island methylation rates in the *SLC6A4* promoter and exposure to early-life adversities [Beach et al., 2010, 2011]. Others reported hypomethylation of *SLC6A4* promoter CpG island in high stress environments [Alasaari et al., 2012] and higher depression levels associated with decreased *SLC6A4* promoter methylation [Devlin et al., 2010]. Subjects experiencing more traumatic events were at increased risk for PTSD only at lower methylation levels, whereas at higher methylation rates, individuals with more traumatic events were protected from this disorder [Koenen et al., 2011]. Also interaction between *SLC6A4* CpG island methylation and 5-HTTLPR genotype was reported to predict unresolved loss or trauma [Van Ijzendoorn et al., 2010].

Within the promoter region of *SLC6A4* and near the CpG island that was shown to be relevant for methylation, another functional element appears notable: the primate specific *Alu*, which belongs to the group of retrotransposons. *Alus* are highly prevalent in the genome, have wide-range influences on gene expression and have been identified to harbor a disease causing effect for more than 60 human diseases [Deininger, 2011; Kaer and Speek, 2013; Kuehnen and Krude, 2012]. They comprise high numbers of CpG sites and are typically highly methylated and thereby silenced via the body's defence mechanisms, whereas decreased or hypomethylation relates to activation and pathophysiology [Bae et al., 2012; Park et al., 2011]. For *SLC6A4* an *Alu* element of subtype *AluJb* is located in the promoter region between 5-HTTLPR and the CpG island (Fig. 1). This *AluJb* contains several CpG

Figure 1.

Functional characteristics of the 5-HTT gene (*SLC6A4*) promoter region. **(A)**: Relative location of predicted (putatively) functional elements according to UCSC genome browser (Human Feb. 2009 Assembly, GRCh37/hg19), indicating a simple tandem repeat (STR) including the functional 5-HTTLPR and rs25531 polymorphisms, our target retrotransposon *AluJb* which belongs to the family of short interspersed nuclear elements (SINE) and

a CpG island overlapping the first exon with 5' untranslated region (5'UTR) and translation start site (TLS). **(B)**: Reference sequence of *AluJb* PCR amplicon with position and sequence of primers in bold letters and position of eight CpG sites (named according to position in amplicon as: bp80, bp86, bp102, bp110, bp161, bp163, bp173, bp177) illustrated by underlined capitals.

sites and might bear the potential to regulate *SLC6A4* gene expression; however, to the best of our knowledge it has never been investigated so far.

As well, previous studies investigating *SLC6A4* methylation rates mainly considered clinical or questionnaire outcomes, leaving underlying neurobiological mechanisms unaddressed. Neuroimaging markers assessing brain structure and function could serve as intermediate phenotypes more closely reflecting effects of (epi-)genetic alterations [Caspi et al., 2010; Caspi and Moffitt, 2006] but to the best of our knowledge, no study hitherto investigated associations between *SLC6A4* promoter methylation rates, particularly for *AluJb*, and neuroimaging markers in humans at all. In the present study, we sought to fill this gap by employing a combined imaging genetics/epigenetics approach.

We selected brain morphology and particularly hippocampal gray matter structure as imaging marker for the association with *SLC6A4* promoter *AluJb* methylation rates since hippocampal atrophy is probably the most frequently replicated imaging marker in depression [Arnone et al., 2011; Cole et al., 2011a; MacQueen and Frodl, 2010], and smaller hippocampal volumes appear to mediate the association of early-life adversity and the onset of depression [Rao et al., 2010]. Furthermore, hippocampal size is highly heritable [Glahn et al., 2012] and affected by 5-HTT genetic variation [Frodl et al., 2008, 2010a]. Finally, the hippocampus is highly susceptible for the detrimental effects of stress or child maltreatment, which in turn were associated with *SLC6A4* methylation rates [Dannlowski et al., 2012; Frodl et al., 2010b; Teicher et al., 2012].

Thus, we hypothesized that *SLC6A4* promoter *AluJb* methylation rates are associated with hippocampal gray matter structure. We further investigated the role of 5-

HTTLPR/rs25531 genotype, based on previous reports regarding a potentially interacting role of genotype with methylation rates.

METHODS AND MATERIALS

Subjects

Two independent samples of $N = 97$ subjects each were enrolled in the present study for a total sample of $N = 194$ right-handed healthy participants. Subjects of both samples responded to local newspaper ads and public notices and were consecutively recruited in the area of Münster by independent teams (sample 1 was recruited in 2009–2010, and sample 2 was recruited in 2010–2011). All subjects were thoroughly investigated by experienced psychologists and free from any life-time history of psychiatric disorders according to DSM-IV criteria [American Psychiatric Association, 1994], as diagnosed with the SCID interview [Wittchen et al., 1997]. Exclusion criteria encompassed any neurological abnormalities, history of seizures, head trauma or unconsciousness, intake of any psychotropic medication, and the usual MRI-contraindications. Five subjects were excluded for strong artifacts or anatomical abnormalities discovered in the structural MRI-images checked by visual inspection and identification as extreme outliers in the check data quality function of the VBM8-Toolbox, leaving $N = 189$ subjects in the full sample ($N = 94$ and $N = 95$ in sample 1 and 2, respectively). Trait anxiety was measured with the State-Trait Anxiety Inventory (STAI, trait version) [Laux et al., 1981] and depression level with the Beck Depression Inventory (BDI) [Beck and Steer, 1987]. Verbal intelligence was estimated by the Mehrfachwahl-Wortschatz-Intelligenztest (multiple choice

TABLE I. Sociodemographic, questionnaire, and genotype data of study participants

	Sample 1 (N = 94)	Sample 2 (N = 95)	P value*
Age	36.9 ± 9.8	34.2 ± 12.0	0.09
Sex (m/f)	42/52	46/49	0.66
5-HTTLPR/rs25531 (L _A L _A /L _A L _G / L _A S _A /L _A S _G /L _G L _G /L _G S _A / L _G S _G /S _A S _A /S _A S _G /S _G S _G)	21/8/39/0/0/4/0/22/0/0	25/3/37/0/2/8/0/20/0/0	0.30
5-HTTLPR/rs25531 risk alleles (0/1/2 L _G or S)	21/47/26	25/40/30	0.55
Verbal IQ ^a	117.8 ± 12.1	117.8 ± 11.7	0.99
Education years	15.7 ± 2.2	15.2 ± 2.2	0.11
STAI-trait ^b	31.7 ± 6.1	32.1 ± 7.4	0.64
BDI ^c	1.4 ± 2.0	2.3 ± 3.5	0.04

*Mean ± SE.

^aAssessed with the Mehrfachwahl-Wortschatz-Intelligenztest (MWT-B[Lehrl, 1995]).^bState-Trait Anxiety Inventory, trait version [Laux et al., 1981].^cBeck Depression Inventory [Beck and Steer, 1987].

vocabulary intelligence test; MWT-B) [Lehrl, 1995]. Table I lists sociodemographic and questionnaire data of study participants. The study was approved by the Ethics Committee of the University of Münster. After complete description of the study to the participants, written informed consent was obtained. Participants received a financial compensation.

DNA Analysis

From all 189 participants, venous blood samples were taken within 30 min after the scanning session. DNA was isolated as recommended (FlexiGene DNA Kit; Qiagen, Germany), dissolved in 25 mM Tris-HCL buffer (pH 7.8) and diluted to 25 ng µl⁻¹.

For analysis of individual methylation profiles, equal amounts of DNA (500 ng) per subject were sodium bisulfite converted in two batches (94 and 95 DNAs of sample 1 and 2, respectively, plus No Template Controls) using EZ DNA Methylation Kit (Zymo Research, HiSS Diagnostics GmbH, Germany) with minor modifications: 5-min incubation was added to step 12 (prior to centrifugation); this modified step 12 was repeated with 12 µl M-Elution Buffer; both eluates were pooled. For PCR-amplification of converted batches, bisulfite sequencing primers were designed using Methyl Primer Express Software v1.0 and Primer Express Software v2.0 (Applied Biosystems by Life Technologies, Germany) and tested for specificity using the BiSearch web server [Arányi et al., 2006; Tusnády et al., 2005]. They encompassed *AluI*b in the *SLC6A4* promoter region (Fig. 1) and were extended by C-rich 5' tails for optimal PCR performance (F-CCCCATGGTTTAAAA TAATAGGTTTTTGTGG, R-GGGGAACAAAACCCCATC TCTAAAAAAA). Standard PCR setup included 30 ng DNA, 0.8 µM primer and 1x ZymoTaq PreMix (Zymo Research) in 20 µl final volumes with PCR conditions of:

initial denaturation (95°C, 10 min), 40 PCR cycles (94.5°C—1 min, 58°C—1 min, 72°C—2 min) and final extension (72°C—7 min). PCR products were cleaned (Vacuum Manifold and MultiScreen HTS Filter Plates, Millipore GmbH, Germany) and used for sequencing reactions with BigDye Terminator chemistry (v3.1 Cycle Sequencing Kit, Applied Biosystems) in 10 µl final volumes of 3 µl PCR product plus 7 µl Mastermix (1 µl BigDye, 2 µl 5× Sequencing Buffer, 0.3 µM primer) with conditions: initial 96°C (1 min) followed by 25 cycles (96°C—10 s, 50°C—4 min). After removal of excess Dye Terminator (Sephadex-MultiScreen-HV plate system, Millipore GmbH) cleaned products were run on a 3730 DNA analyzer (Applied Biosystems). Electropherograms were manually checked for fluorescence intensities, overall quality and analyzability of CpG sites using sequence scanner software v1.0 (Applied Biosystems). Quantitative analysis of CpG site specific methylation (relative peak heights C/C+T) was performed with ESME software including quality control, correction for incomplete bisulfite conversions, normalization of signals and alignment of own generated sequence trace files and reference sequences (public databases) as recommended [Lewin et al., 2004] and described previously [Domschke et al., 2012b]. Standard PCR and sequencing reactions were repeated independently for quality control: duplicate data were controlled for concordance (SD of mean CpG site specific methylation rate per proband to be ≤ 0.05) or further independent sequencing reactions were performed. Only mean data with SD ≤ 0.05 were included in further analysis (see below). In addition, seven random proband DNAs were employed to three or four independent conversions with sodium bisulfite followed by independent standard PCRs and sequencing reactions for each converted sample up to three times. The resulting independent sequence data sets (2 DNAs: 4 sets, 2 DNAs: 6 sets, 1 DNA: 8 sets, 2 DNAs: 12 sets)

revealed high concordance rates at all CpG sites with SD of mean methylation rates within the same DNA to be <0.05 in all cases.

Genotyping of the functional promoter polymorphisms 5-HTTLPR (long (L) and short (S) allele) and rs25531 (common A and rare G allele) was performed according to already published protocols [Baune et al., 2008; Deckert et al., 1997; Wendland et al., 2006] with minor modifications. Briefly, primers F-CAGCAACTCCCTGTACCCCTCCTAGGATC and R-GATGCGGGGAATACTGGTAG-GTGC (10 pM each) were used for 20 μ l PCRs containing 60 ng DNA, 200 μ M dNTPs, and 0.5 U HotStar Taq Polymerase with 1.5 mM $MgCl_2$, 1xQ-Solution and 1xBuffer (Qiagen). Initial denaturation (95°C, 15 min) preceded 35 PCR cycles (94°C—1 min, 68°C—1 min, 72°C—2 min), and final extension (72°C, 10 min) and resulted in PCR products of 279 bp (S allele) or 322 bp (L allele). 5-HTTLPR-rs25531 haplotype determination was based on restriction fragments of 61 to 261 bp length: $L_A L_A$ (261+61), $L_A L_G$ (261+174+87+61), $L_G L_G$ (174+87+61), $S_A S_A$ (218+61), $S_A S_G$ (218+131+87+61), $S_G S_G$ (131+87+61), $L_A S_A$ (261+218+61), $L_A S_G$ (261+131+87+61), $L_G S_A$ (218+174+87+61), $L_G S_G$ (174+131+87+61). Subjects with the genotypes $L_A L_G$, $L_A S_A$ or $L_A S_G$ were assigned to the group carrying one risk allele (S or L_G) and subjects with the genotypes $L_G L_G$, $L_G S_A$, $L_G S_G$, $S_A S_A$, $S_A S_G$, $S_G S_G$ were assigned to the group carrying two risk alleles.

All quantitative analyses of individual methylation profiles and genotyping analyses were performed by investigators blind for subject characteristics or structural imaging data.

Voxel-based Morphometry

T1 weighted high resolution anatomical images were acquired with a 3D fast gradient echo sequence ("Turbo Field Echo", TFE), TR = 7.4 ms, TE 3.4 ms, FA = 9°, with cubic voxels of 0.5 mm edge length. The VBM8-toolbox (<http://dbm.neuro.uni-jena.de/vbm>) was used for preprocessing the structural images with default parameters. Images were bias-corrected, tissue classified, and normalized to MNI-space using linear (12-parameter affine) and nonlinear transformations, within a unified model [Ashburner and Friston, 2005] including high-dimensional DARTEL-normalization. Gray and white matter segments were modulated only by the nonlinear components in order to preserve actual GM and WM values locally (modulated GM and WM volumes).

Homogeneity of gray matter images was checked using the covariance structure of each image with all other images, as implemented in the check data quality function. As described above, five extreme outliers showing anatomical abnormalities or artifacts were identified and excluded. The remaining $N = 189$ images were clear of such problems. The modulated gray matter images were

smoothed with a Gaussian kernel of 8 mm FWHM. Group statistics were calculated using SPM8.

First, we employed a voxel-wise regression model restricted to the bilateral hippocampus and parahippocampal gyrus as defined by Tzourio-Mazoyer et al. [2002], to predict hippocampal gray matter volume by *SLC6A4 Alu/b* methylation rates. Because gray matter volume of the anterior cingulate gyrus [Frodl et al., 2008; Pezawas et al., 2005] and amygdala function [Domschke et al., 2012a; El-Hage et al., 2013; Murphy et al., 2013] have also been associated with *SLC6A4* genotype in previous work, we have incorporated these areas as well. The methylation rates of all eight investigated sites were highly intercorrelated (see results below) and therefore, we used the mean methylation rate of all sites as an estimate of *SLC6A4* promoter *Alu/b* methylation status. The analysis was conducted in both samples separately and furthermore in the combined sample. To control for multiple statistical testing within our region of interest (ROI), we maintained a cluster-level false-positive detection rate at $P < 0.05$ using a voxel-level threshold of $P < 0.01$ with a cluster extent (k) empirically determined by Monte Carlo simulations ($n = 1,000$ iterations), as reported in our previous publications [Baune et al., 2012a; Baune et al., 2012b; Dannlowski et al., 2012]. This was performed by means of the AlphaSim procedure which accounts for spatial correlations between GM values in neighboring voxels [Forman et al., 1995], implemented in the REST toolbox (<http://restfmri.net/forum/index.php>). The empirically determined cluster threshold for the bilateral hippocampus, ACC, and amygdala mask was $k = 186$ voxels. The combined sample had sufficient power ($1-\beta = 80\%$) to detect relatively small effect sizes ($r = 0.12$).

Further analyses were conducted to investigate effects of methylation rates across the entire brain, and to explore effects of 5-HTTLPR genotype and other covariates of interest. These supplementary analyses were conducted in the entire sample to achieve sufficient statistical power. Our whole-brain analysis correlating methylation rates with gray matter volume was performed at a rigorous threshold of $P < 0.05$, FWE-corrected for the entire brain. The anatomical labeling for the whole-brain data was performed by means of the AAL-Toolbox [Tzourio-Mazoyer et al., 2002], and the Brodmann areas (BA) were identified with the Talairach Daemon atlas (<http://www.talairach.org>).

The effects of 5-HTTLPR genotype, including rs25531 (triallelic model) on hippocampal gray matter volume was investigated using two separate factorial models. A one-factor between-subjects ANOVA with genotype group (carriers of 0 vs. 1 vs. 2 risk S or L_G alleles) was employed to investigate the main effects of genotype. In a second model, mean methylation rate was added as interacting covariate to the previous model and the interaction term of genotype group and methylation rate was observed. These models were conducted at the same corrected statistical threshold as in our previous ROI analysis ($P < 0.01$, $k = 186$).

Figure 2.

Association of mean 5-HTT gene (*SLC6A4*) promoter *AluJb* methylation rates and hippocampal gray matter volumes in the entire sample. Left: Results from a ROI-analysis of the bilateral hippocampus including the parahippocampal gyrus, conducted at $P < 0.01$, $k = 131$ (resulting in a cluster-corrected $P < 0.05$). Color

bar, correlation coefficient r . Right: Scatter plot depicting the association between mean *AluJb* methylation rate and gray matter volume extracted from the peak voxel in the right hippocampus ($x = 18$, $y = -4$, $z = -12$, $r = 0.36$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Finally, we investigated whether other factors influenced the association of methylation rates and gray matter structure by adding age, gender, BDI, STAI-trait, education years, and verbal IQ as nuisance covariates.

RESULTS

Methylation Rates

All eight selected CpG sites showed differential methylation rates (mean $\pm$ SD: bp80 $44.4\% \pm 5.4\%$, bp86 $40.3\% \pm 5.1\%$, bp102 $17.5\% \pm 3.7\%$, bp110 $43.0\% \pm 6.6\%$, bp161 $40.1\% \pm 5.9\%$, bp163 $63.0\% \pm 6.0\%$, bp173 $68.7\% \pm 4.4\%$, bp177 $67.0\% \pm 6.1\%$) which were approximately normally distributed, according to Kolmogoroff-Smirnov Tests (all $P > 0.066$) and which were highly inter-correlated (all $r > 0.297$, all $P < 0.001$). Hence, we used the mean methylation rate of all investigated sites as an estimate of *SLC6A4* promoter *AluJb* methylation status. For a distribution of genotypes see Table I. According to a one-factorial ANOVA, genotype group (0 vs. 1 vs. 2 risk alleles) was not associated with mean methylation rate, $F(2,188) = 0.65$, $P = 0.53$ or with methylation rates at any of the single CpG-sites (all $P > 0.12$).

VBM Results

The ROI analysis of the bilateral hippocampus, ACC and amygdala revealed a significant positive association of

bilateral hippocampal gray matter volume and mean *AluJb* methylation rates in sample 1 (left: $x = -15$, $y = -18$, $z = -23$, $t(92) = 4.86$, $P < 0.00001$, $k = 2281$, $r = 0.45$; right: $x = 18$, $y = -18$, $z = -23$, $t(92) = 4.05$, $P = 0.00005$, $k = 1915$, $r = 0.39$) and in sample 2 (left: $x = -12$, $y = -37$, $z = 9$, $t(93) = 3.56$, $P = 0.00029$, $k = 878$, $r = 0.35$; right: $x = 14$, $y = -36$, $z = 6$, $t(93) = 4.08$, $P = 0.00005$, $k = 632$, $r = 0.39$). In both samples, peak coordinates were found in the bilateral hippocampus. In sample 1, the clusters also extended to the bilateral amygdala, which was not found in sample 2. There was no association of ACC volumes with methylation rate in any sample.

Unsurprisingly, this association was found also in the entire sample (left: $x = -28$, $y = -25$, $z = -9$, $t(187) = 4.71$, $P < 0.00001$, $k = 2,976$, $r = 0.33$; right: $x = 18$, $y = -4$, $z = -12$, $t(187) = 5.22$, $P < 0.00001$, $k = 2,774$, $r = 0.36$, Fig. 2), with peak voxels located in the bilateral hippocampus extending to the amygdala. Calculating the association of hippocampal gray matter volume and methylation rate of all eight sites separately revealed that each single site produced a significant or near significant result in the bilateral hippocampus (smallest $k = 164$ voxels for bp102 in the right hippocampus).

Whole Brain Analysis

There were significant clusters in the bilateral hippocampus positively associated with mean methylation rate,

TABLE II. Results of a whole-brain regression analysis of mean *SLC6A4* promoter *AluJb* methylation rates on gray matter volumes conducted at $P < 0.05$, FWE-corrected for the entire brain

Anatomical region	Side	Cluster size	<i>x</i>	<i>y</i>	<i>z</i>	Z-score	P-value (FWE)
Insula, putamen	R	309	34	14	-9	5.17	0.003
Hippocampus, amygdala	R	54	18	-4	-12	5.04	0.006
Superior occipital gyrus	L	8	-14	-93	6	4.81	0.017
Caudate	R	84	14	12	15	4.69	0.029
Insula	L	7	-38	15	-6	4.62	0.038
Hippocampus	L	3	-28	-24	-9	4.58	0.044

Coordinates are given in MNI space.

even at this rigorous threshold corrected for the entire brain. Further areas showing effects in the same direction included the bilateral insula, right amygdala and putamen, left caudate and superior occipital gyrus (see Table II for details). There were no brain areas showing a negative correlation between methylation rates and gray matter volume.

Effects of 5-HTTLPR/rs25531 Genotype

There was no main effect of genotype group on hippocampal gray matter structure, even after reducing the statistical threshold to an overly lenient threshold ($P < 0.01$, $k = 10$). Furthermore, there was no significant interaction of genotype $\times$ mean methylation rate ($k = 125$). Accordingly, the positive association between methylation rates and hippocampal gray matter density was found in carriers of two risk alleles ($N = 56$) (left: $x = -16$, $y = -4$, $z = -12$, $t(181) = 4.00$, $P = 0.00005$, $k = 461$, $r = 0.28$; right: $x = 18$, $y = -13$, $z = -18$, $t(181) = 3.25$, $P = 0.00069$, $k = 338$, $r = 0.23$) and in carriers of one risk allele ($N = 87$) (left: $x = -36$, $y = -16$, $z = -12$, $t(181) = 3.70$, $P = 0.00014$, $k = 303$, $r = 0.27$; right: $x = 18$, $y = -6$, $z = -14$, $t(181) = 3.92$, $P = 0.00006$, $k = 817$, $r = 0.28$). However, in the smaller subsample of $L_A L_A$ homozygotes ($N = 46$) this association did not reach a corrected level of significance, albeit pointing to the same direction ($x = 24$, $y = 9$, $z = -23$, $t(181) = 3.06$, $P = 0.0013$, $k = 54$, $r = 0.22$).

Other Covariates

Adding age, gender, education years, IQ, BDI, and STAI-trait as nuisance regressors only marginally reduced the strength of association between mean *SLC6A4* promoter *AluJb* methylation rate and gray matter volume with maximal t values in the right hippocampus of $t = 4.53$ (5.22 without these covariates), left $t = 3.97$ (4.71 without covariates).

DISCUSSION

In the present study, we found a strong association between *SLC6A4* promoter *AluJb* methylation rates and

hippocampal gray matter volumes in two independent samples of healthy subjects. In the combined sample, this association even survived the most conservative alpha-correction for the entire brain and was not affected by age, gender, education, intelligence or subclinical depression and anxiety levels. The 5-HTTLPR/rs25531 genotype showed no significant association with hippocampal gray matter volume, and methylation effects on gray matter volumes appeared to be comparable between the genotype groups.

Our data are in line with previous studies indicating specific DNA methylation pattern within the *SLC6A4* promoter [Alasaari et al., 2012; Beach et al., 2010, 2011; Devlin et al., 2010; Koenen et al., 2011; Van Ijzendoorn et al., 2010;], underscoring the general susceptibility of this region for differential methylation. With the *AluJb* element our study maybe adds a new element to the already known functional players in the *SLC6A4* promoter, because an increasing number of studies suggest the great potential of *Alu* elements to influence nearby gene expression [Deininger, 2011; Kaer and Speek, 2013; Kuehnen and Krude, 2012]. Previous reports related *Alu* hypomethylation to pathophysiology, e.g. carcinogenesis [Bae et al., 2012; Park et al., 2011], response to potential hazardous pollutants [Tarantini et al., 2009], or major psychosis [Kan et al., 2004]. Accordingly, we observed lower *AluJb* methylation associated with lower hippocampal gray matter volumes. Such lower *Alu* methylation might influence nearby gene expression via different mechanisms. First, *Alu* elements are predestined to provide alternative exons, the so called exonization [Sela et al., 2007], especially so around the transcription start site of nearby genes [Sela et al., 2010], and when oriented in antisense direction to them [Schmitz and Brosius, 2011], as it is the case in the presently studied *AluJb*. As a result of this process, novel protein isoforms might be created that compete against or even replace the original protein [Schmitz and Brosius, 2011], in particular in the evolutionary early subfamilies as shown for an *AluJb* element in the leptin receptor gene [Huh et al., 2010]. Thus, high *SLC6A4* promoter *AluJb* methylation should prevent the synthesis of such alternative proteins whereas lower methylation could lead to synthesis of them combined with lower amount of original

5-HTT due to competitive transcription. Lower 5-HTT levels as shown for the 5-HTTLPR/rs25531 S/L_G-alleles, finally, have been associated with a broad range of alterations [Clarke et al., 2010; Dannlowski et al., 2007, 2008, 2010; Miller et al., 2013; Munafò et al., 2008; Pergamin-Hight et al., 2012], including reduced hippocampal volumes [Frodl et al., 2008, 2010a]. Second, *Alu*s contain a number of transcription-factor-binding sites (TFBS) [Deininger, 2011], particularly so the evolutionary old members like *AluJ* [Oei et al., 2004; Polak and Domany, 2006]. Indeed, for the *SLC6A4* promoter *AluJb* sequence (Fig. 1B), 63 TFBS were predicted (MatInspector Release professional 8.06 [Cartharius et al., 2005]), of which 38 overlapped at least one CpG site and six included a CpG site within its functionally most relevant (core) sequence: AML3, ATF6, KLF7, PAX6, TFIIC, and ZBRK1. PAX6 (Paired box protein Pax-6) is of particular importance as it is not only assumed in brain and CNS development but also was experimentally found to bind to specific *Alu* sequences [Polak and Domany, 2006]. If this might also be true for the *SLC6A4* promoter *AluJb*, then lower methylation would allow better PAX6 binding resulting in enhanced repression of *SLC6A4* gene expression and thus lower 5-HTT levels with the above assumed alterations [Clarke et al., 2010; Frodl et al., 2008, 2010a]. However, both considerations are speculative since no experimental data exist for this specific gene region so far. It would be of interest to extend our investigations on *SLC6A4* expression pattern in subjects characterized for both, hippocampal volumes and DNA methylation pattern, to confirm or disconfirm our considerations. An alternative possibility might be that the *AluJb* methylation has no direct functional consequences on *SLC6A4* gene expression but rather reflects other truly functional variants in or nearby the gene that mediate gene expression and hippocampal volumes.

We found lower methylation rates to be associated with smaller hippocampal volumes, which is a frequent imaging finding in major depression and other neuropsychiatric disorders [MacQueen and Frodl, 2010]. Because reduced hippocampus size has already been reported in subjects at genetic or environmental risk for depression [Amico et al., 2010; Chen et al., 2010; Rao et al., 2010], it was argued that smaller hippocampal volumes might rather constitute a risk factor for than a feature of depression [Amico et al., 2010]. Accordingly, it was reported that reduced hippocampal volumes could act as a mediator for the relation between early-life stress and depression onset [Rao et al., 2010]. Hence, *SLC6A4* promoter *AluJb* methylation might increase the susceptibility for depression and other stress-related disorders by altering hippocampal gray matter structure. However, since we did not investigate patients suffering from neuropsychiatric disorders so far, this remains to be investigated in future studies including patient samples.

In addition to hippocampal gray matter changes, similar associations of methylation rates were detected in the amygdala, insula, and the striatum. Interestingly, accord-

ing to neurobiological models of affective disorders, these areas are key players in the etiology of depression and belong to a ventral system crucially involved in the identification of the emotional significance of incoming stimuli and the production of affective states [Drevets et al., 2008; Phillips et al., 2003]. The amygdala ranks among the most frequently investigated structure in neuroimaging studies on depression and several studies found functional and structural aberrations in patient samples [Bora et al., 2011; Stuhrmann et al., 2011, 2013; Suslow et al., 2010]. The ventral striatum, including the caudate nucleus and putamen, plays a crucial role in reward processing. Accordingly, structural deficits have been meta-analytically confirmed in depression [Arnone et al., 2011], which might be associated with impaired reward processing in these patients [Nestler and Carlezon, 2006]. Finally, also the insula, and particularly the anterior parts, have been frequently reported to show volume reductions in samples of depressed patients [Bora et al., 2011; Sprengelmeyer et al., 2011; Takahashi et al., 2010], probably associated with deficits in emotion perception [Sprengelmeyer et al., 2011] and regulation [Takahashi et al., 2010]. The remarkable overlap of brain structural alterations associated with *SLC6A4* methylation pattern and major depression might add further support for the role of *SLC6A4* gene expression in the etiology of affective disorders.

It remains unclear how changes of gene transcription can change the structure of these brain regions, but several studies suggested explanations. Reduced 5-HTT availability and function due to lowered *SLC6A4* transcription levels and a therefore reduced serotonin uptake capacity increases serotonin neurotransmission [Lesch et al., 1996]. Such changes in serotonin levels possibly influence (early) neurodevelopmental processes and, as a consequence of this, changes of gray matter volumes, neuronal circuitries, and connectivity between brain regions involved in emotion processing are supposable and could increase the risk for affective disorders [Caspi et al., 2010; Frodl et al., 2008; Lesch et al., 1996; Pezawas et al., 2005].

Our negative finding regarding the association of 5-HTTLPR/rs25531 genotype and hippocampal gray matter volumes is in line with a recent report failing to replicate this association in a larger sample [Cole et al., 2011b] in contrast to earlier reports [Frodl et al., 2008; Frodl et al., 2010a]. The present finding might add to the notion that epigenetic processes like DNA methylation might exert stronger neurobiological effects than genotypic variance. Thus, our sample could be still underpowered to detect effects of genotype on hippocampal volumes. Further investigations in subsequent, preferably larger samples are highly warranted.

Our study has strengths and limitations. First of all, we used DNA isolated from peripheral EDTA-blood, which does not allow for direct conclusions regarding methylation patterns in brain tissue. However, peripheral patterns have been suggested to reflect central processes [Domschke et al., 2012b; Mill and Petronis, 2007] and

5-HTT promoter methylation rates measured in peripheral blood cells were reported to be linked to central *in vivo* 5-HT synthesis as detected by PET [Wang et al., 2012]. Second, we designed PCR primers to cover most *AluIb* CpG sites (eight of nine), but also other nearby sites not investigated here could be biologically relevant. Alasaari et al. [2012] investigated five adjacent CpG sites (about 250 bp away in 3' direction) and associated lower methylation levels with high stress environment. Yet this finding might also be explained by the spreading of methylation/demethylation events around *Alu* sites directed to regulate them [Kuehnen and Krude, 2012]. Third, other functional polymorphisms, as well as other genes, might interactively interfere but also additional sociodemographic and environmental factors not tested for in our approach (e.g., family history of affective disorders) might potentially confound our results. For example smoking status was shown to be associated with altered methylation rates in the monoamine oxidase A gene [Philibert et al., 2010], however, not with *Alu* methylation in another study [Zhu et al., 2012]. Furthermore, we failed to obtain measures of early-life stress in our samples and therefore, our notions regarding the association of early-life stress, methylation rates and hippocampal structure remain speculative. Methylation rates did not show significant (but nominally positive) associations with BDI or STAI scores ($r = 0.11$, $P = 0.14$ and $r = 0.07$, $P = 0.31$, respectively). However, in our healthy sample, the distribution of these questionnaire measures had just very little variance to explain (floor effect), e.g., the mean BDI score was just 1.8. Future studies should also investigate clinical samples or healthy samples with more heterogeneity regarding subclinical symptoms.

Despite such limitations, our study provides strong hints towards the impact of *AluIb* methylation on hippocampal gray matter structure and suggests feasible ways of regulating *SLC6A4* expression involving exonization or transcription factor binding as potentially underlying mechanisms.

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