DNA methylation of the serotonin transporter gene (*SLC6A4*) is associated with brain function involved in processing emotional stimuli

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Background: The aim of the present study was to investigate the association of fMRI blood oxygen–level dependent (BOLD) reactivity with the level of epigenetic methylation of *SLC6A4* in blood DNA from a sample of healthy participants and patients with major depressive disorder (MDD). **Methods:** We investigated patients with MDD and healthy controls using fMRI and an emotional attention-shifting task. We assessed site-specific DNA methylation of a previously characterized *SLC6A4* region in peripheral blood DNA using pyrosequencing. **Results:** Our study involved 25 patients with MDD and 35 healthy controls. Activation in the anterior insula elicited by negative emotional content was significantly positively associated with the degree of *SLC6A4* methylation. Significantly negative associations were observed between activation in the posterior insula and the degree of *SLC6A4* methylation when judging the geometry of pictures after seeing negative in contrast to positive emotional stimuli. Healthy controls with a high degree of *SLC6A4* methylation depicted significantly more activity elicited by positive stimuli in limbic regions and more activity elicited by negative stimuli in limbic as well as cognitive control regions than those with a low degree of *SLC6A4* methylation. **Limitations:** It is impossible to measure methylation directly in the brain and thus we assessed peripheral methylation of *SLC6A4*. Since the association was cross-sectional, no conclusion about cause and effect can be drawn. **Conclusion:** Our study provides further support to the hypothesis that particular DNA methylation states that are associated with brain function during emotion processing are detectable in the periphery.

Introduction

Childhood adversity, such as childhood maltreatment, plays an important role in a number of multifactorial mental disorders. Recent studies reveal that certain aspects of stress-related mental disorders result from maladaptive, stress-induced neuroplastic changes in specific neural circuits.[1] Based on fMRI studies, childhood maltreatment seems to result in blunted prefrontal cortical activation when processing higher-order cognitive functions.[2] On the other hand, childhood maltreatment has been reported to result in heightened neural response to emotion processing; however, studies are controversial with regards to their specificity for valences. It was reported that childhood maltreatment was associated with increased neural responses in the amygdala to presentation of both angry and happy emotional expressions in one study[3] and to presentations of only sad facial expressions in another study.[4] In combination with genetic factors, such childhood maltreatment–induced alterations might set the stage for the development of psychopathology. In support of this suggestion, we demonstrated previously that childhood adversity interacts with genetics and affects hippocampal volumes in patients with major depressive disorder (MDD).[5]

Major depressive disorder is one of the most prevalent and burdensome of all psychiatric illnesses associated with early stressors.[6,7] In functional models of the disease, overactivity in limbic areas like the amygdala, anterior cingulate cortex and hippocampus is not adequately controlled by prefrontal areas.[8] These findings share common aspects with those reported from studies on childhood maltreatment. The involvement of

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these brain regions in MDD is supported by a recent meta-analysis.[9] Interestingly, brain function in individuals with MDD was found to be influenced by genetic variants as well.[10] Most evidence comes from genes involved in the regulation of the serotonin (5-HT) system, especially with regard to the 5-HT transporter gene *SLC6A4*. For instance, in a study involving 28 patients with MDD, short allele carriers of the promoter polymorphism of the serotonin transporter gene, commonly known as 5-HTTLPR were found to have increased amygdala reactivity to masked emotional faces.[11]

Thus, stressful environmental and genetic factors affect brain function and play a role in MDD. In particular, gene × environment interactions seem to be highly important. Although the specific mechanisms remain unknown, a number of studies suggest that DNA methylation may be an underlying mechanism mediating the impact of adverse social environments on gene function.[12,13] Studies in patients with MDD have shown an association between differential DNA methylation in white blood cells and early maltreatment as well as depressive symptomatology,[12,14] though not consistently; however, some of the inconsistent effects might be due to differences in methodology.[12] Moreover, peripheral *SLC6A4* DNA methylation was found to be related to childhood maltreatment in a sample of pregnant women and a sample of adoptees.[15–18] A study on prenatal and postnatal exposure to maternal depression reported that increased depressed mood in pregnant women during the second trimester was associated with decreased maternal and infant *SLC6A4* promoter methylation.[17] This correlational finding seems to be in the opposite direction than research on childhood adversity, warranting further research to understand the impact of *SLC6A4* methylation and its function from a developmental point of view.

The functional relevance of DNA methylation in *SLC6A4* promotor regulation was demonstrated by an in vitro experiment, which showed that DNA methylation of the SLC6A4 promoter in a luciferase reporter construct suppressed its transcriptional activity.[19] It was anticipated that DNA methylation states would exhibit cell type specificity and that DNA methylation changes relevant to brain function would be detected only in the brain. Nevertheless, we have previously reported differential methylation of a regulatory region of the *SLC6A4* gene in peripheral T cells associated with differences in in vivo measures of lower 5-HT synthesis measured with positron emission tomography[19] and in hippocampal volume detected by MRI in patients with MDD and healthy controls.[20] Moreover, an association between peripheral *SLC6A4* methylation and several grey matter structures, including the hippocampus, insula, amygdala and caudate nucleus, has been reported.[21] These previous studies suggest that peripheral *SLC6A4* methylation may be a peripheral representative of an underlying epigenetic mechanism through which gene and environment interact in the development of 5-HT-associated stress-related psychopathology. Interestingly, in a postmortem study, significant correlations between individual DNA methylation differences in the blood and those in cortical (r = 0.66, $p <$ 0.001) and cerebellar brain regions (r = 0.76, p < 0.001) were detected.[22] However, a possible involvement of differential DNA methylation of the *SLC6A4* gene in neural regulation of emotions measured with fMRI is not known.

Methylation of the catechol-O-methyltransferase (COMT) Val(158) allele in a CpG site measured in peripheral blood mononuclear cells has been found to be negatively associated with prefrontal cortex fMRI BOLD response during working memory performance in 19 healthy participants homozygous for the Val allele.[23] This specific CpG site, which was the only one showing significance in that study, was seen only in the Val allele and thus the number of participants in this group for correlation was very small. Thus, the study reported promising results, but investigations in larger and clinical samples are necessary. Recently, increased promoter methylation of the serotonin transporter gene predicted increased threat-related amygdala reactivity in healthy participants, providing further evidence for an association between peripheral measurement of epigenetics and brain function.[24] The study used saliva-derived DNA from a discovery cohort of 80 young adults and blood-derived DNA from an independent replication cohort of 96 adolescents and focused on amygdala activation.

Previously, we reported an interactive effect between the promotor polymorphism of *SLC6A4* and childhood adversity on brain structure specifically in patients with depression and not in healthy controls,[5] suggesting diagnosis-specific epigenetic regulatory effects. The aim of the present study was thus to investigate in a new sample using functional imaging whether the methylation state of a previously characterized differentially methylated regulatory region of the *SLC6A4* gene in whole blood DNA might be associated with frontolimbic neural regulation of emotions and how specific such an effect might be in the patient group. We hypothesized based on knowledge from studies in the areas of MDD and childhood maltreatment that higher peripheral *SLC6A4* DNA methylation is associated with lower brain reactivity in higher-order cognitive processes and increased limbic–paralimbic reactivity elicited by emotional stimuli. Moreover, we examined interactive effects on brain BOLD responses between diagnosis of MDD and methylation of *SLC6A4* as well as differences between patients with MDD and healthy controls in order to explore whether effects of methylation of *SLC6A4* affect brain function to a larger extent in patients than controls. This idea stems from our previous research and the fact that methylation of *SLC6A4* might be more pronounced in patients with MDD.[12,14] Here we focus on DNA methylation in that region of the SLC6A4 promoter and associated specific CpG sites that was previously most strongly associated with in vivo measures of lower 5-HT synthesis.[19]

Methods

Participants

Few recruited adult patients with MDD from the mental health services of Tallaght Hospital or St. James's Hospital, Dublin, Ireland. Patients had a clinical diagnosis of MDD based on DSM-IV criteria and confirmed by an independent psychiatrist using the Structured Clinical Interview for DSM-IV Axis I

Disorders (SCID). We recruited healthy controls from the local community, and the groups were balanced for age and sex. Exclusion criteria were age younger than 18 or older than 60 years, history of neurologic or comorbid psychiatric disorders (Axis I or Axis II), other severe medical illness, head injury or substance abuse. We also excluded patients taking antipsychotics or mood stabilizers. Demographic characteristics and inclusion and exclusion criteria were obtained through a structured interview by a psychiatrist and documented using a standardized questionnaire. Patients with MDD were all ex-periencing acute depressive episodes and were either currently medication-free or taking an antidepressant. Thus it was possible to control for medication effects.

We obtained written informed consent from all participants after they were given a detailed description of the study, which was designed and performed in accordance with the ethical standards laid out by the Declaration of Helsinki and was approved by the ethics committee of St. James's and Tallaght Hospitals, Dublin, Ireland, as well as McGill University, Montréal, Canada, and Queen's University, Kingston, Canada.

Rating instruments

Self- and observer-rated scales were administered for all participants; these included the Hamilton Rating Scale for Depression (HAM-D),[25] Beck Depression Inventory (BDI-II),[26] and the Structured Clinical Interview for DSM-IV (SCID-II) personality questionnaire.[27] We also administered the Childhood Trauma Questionnaire (CTQ),[28] which is a standardized self-report instrument that assesses 5 types of childhood maltreatment: emotional, physical and sexual abuse and emotional and physical neglect. Reliability and validity of the CTQ have been established, including measures of convergent and discriminative validity from structured interviews, stability over time and corroboration.[29] Childhood maltreatment was calculated as the sum score of all 5 categories.

MRI data acquisition

We obtained MRI scans with a Philips Achieva 3 T MRI scanner. The protocol consisted of the acquisition of a high-resolution, 3-dimensional T_1-weighted structural data set (spoiled gradient recalled acquisition sequence with the following parameters: repetition time [TR] 8.5 ms; echo time [TE] 3.9 ms; fields of view [FOVs] foot to head 256 mm, anterior to posterior 256 mm, right to left 160 mm; matrix 256×256; spatial resolution 1 mm^3), followed by an fMRI experiment (spin-echo echo-planar imaging sequence with the following parameters: TR 2000 ms, TE 35 ms, in plane resolution 3×3 mm^2, 4.8 mm slice thickness, 550 dynamic scans each with a duration of 2 s).

Functional MRI

An established visual emotional attention-shifting task was used in the fMRI experiment (see the Appendix, available at jpn.ca).[30] The task was event-related and consisted of 180 pseudorandomized trials. Each trial in the task lasted 4 s and consisted of viewing an emotional picture taken from the International Affective Picture System (IAPS) database and answering a question about the emotional valence or the shape of the pictures. Participants had to either focus on the emotion of the picture and answer whether this was positive, negative or neutral, or they had to shift their attention away from the emotion and answer a question about the shape of the picture (horizontal v. vertical).

Behavioural data were analyzed using the Analyze module in Presentation software. The time that participants took to answer the questions about the shape or the emotional content of the picture and the accuracy of their responses (the number of incorrect responses) were analyzed separately for each emotional condition. Thus, different valence judgment of positive, negative or neutral images was counted.

Preprocessing steps for fMRI data are described in the Appendix and elsewhere,[30] and resulted in the following contrasts: positive or negative picture stimuli versus neutral picture stimuli, shifting attention away from negative stimuli versus with shifting attention away from positive stimuli, and judging the emotional content versus judging the geometry of the images for each emotional valence separately.

DNA methylation

Pyrosequencing

We assessed DNA methylation from whole blood DNA using our assay, as previously validated and applied in T cells and monocytes DNA.[19] In the present study we used whole blood DNA and hypothesized that differential DNA methylation would be detectable in whole blood DNA as it was in selected white blood cell subtypes. We targeted CpG sites 5–15, because CpG sites within this region were previously most strongly associated with in vivo measures of brain 5-HT synthesis[19] and thus most relevant to test our current hypotheses. The methylation percentages at each CpG site were analyzed using the PyroMark Q24 software (Qiagen) and determined in triplets. Quality control was carried out to ensure correct analysis. For the association analysis with fMRI we used the mean of methylation percentage from sites 5–15 (see the Appendix for further details).

Statistical analysis

All results of our statistical analyses were considered to be significant at $p < 0.05$. We tested differences in demographic variables using a Student t test or a χ^2 test in case of categorical variables. We performed a regression analysis with methylation of *SLC6A4* and childhood maltreatment, diagnosis, age and sex as predictors. Response times and accuracy of responses during the task were subjected to a 2×2 analysis of covariance (ANCOVA) with group (MDD v. control) and methylation of *SLC6A4* (high v. low) as factors, and age and sex as cofactors. The definitions of high and low were based on a median split for percentage methylation of *SLC6A4*, with high defined as 3.8% or higher across the investigated region and low defined as less than 3.8% across the investigated region.

For fMRI analyses we performed a 2×2 ANCOVA with SPM8 on the contrasts where the first factor was group

(MDD v. control) and the second factor was methylation of *SLC6A4* (high v. low), while age, sex and medication were used as covariates. Whole brain cluster level family-wise error (FWE) correction with $p < 0.05$ was used in all comparisons to ensure statistical significance of our findings. We used the automated anatomic labelling atlas to localize the significant areas in a standard stereotactic space (template from the Montreal Neurological Institute [MNI]). Significant clusters were then extracted for further statistical analysis. Kolmogorov–Smirnov tests revealed that both the extracted fMRI BOLD responses and the *SLC6A4* methylation did not differ from a normal distribution. Thus, we ran Pearson correlations to test for linear associations between methylation of *SLC6A4* and brain function as well as response times and accuracy of responses, whereby a Bonferroni correction for multiple testing was applied.

Results

For this study, 60 adult participants were included. The sample comprised 25 adult patients with MDD and 35 healthy controls, and the groups were balanced for age and sex (Table 1). Patients with MDD were all experiencing acute depressive episodes and were either currently drug-free or on an antidepressant. The patients and controls were well matched, with no significant between-group differences found for demographic data (Table 1). The regression model including age, sex and childhood maltreatment as predictors explained 15.4% variance of *SLC6A4* methylation ($F = 3.6$, $p = 0.012$). Greater childhood maltreatment was associated with greater percentage of *SLC6A4* methylation ($\beta = 0.37$, $p = 0.012$). Patients, as expected, displayed more childhood maltreatment than healthy controls (mean 44.5 ± 18.2 v. 30.2 ± 4.9, $t = 3.8$, $p = 0.001$). Older age was associated with *SLC6A4* methylation at a trend level ($\beta = 0.23$, $p = 0.08$). Diagnosis ($p = 0.38$) and sex ($p = 0.11$) were not found to be significantly associated with *SLC6A4* methylation.

Behavioural parameters

Interestingly, patients with MDD judged positive stimuli as not positive and neutral stimuli as not neutral significantly more often than controls ($F_{1,54} = 15.1$, $p < 0.001$ for positive, $F_{1,54} = 8.5$, $p = 0.005$ for neutral). Patients with MDD took more time than controls to respond (Appendix, Table S1). No significant differences in mean reaction times and numbers of incorrect valence judgment were found between participants with high versus low *SLC6A4* methylation, nor were there any significant interactions between group (MDD v. controls) and methylation (high v. low) on these response parameters. No significant correlations between methylation of *SLC6A4* and behavioural data remained significant after Bonferroni correction.

Functional MRI BOLD signal

Overall effect of DNA methylation

When viewing negative picture stimuli in contrast to neutral picture stimuli, participants with higher percentage of *SLC6A4* methylation activated the left anterior insular cortex and left frontal inferior operculum significantly more than participants with lower percentage of *SLC6A4* methylation ($p = 0.002$, FWE-corrected).

Table 1: Demographic and clinical data for patients with major depressive disorder and healthy controls as well as for participants with low and high methylation of *SLC6A4*

	Group, mean ± SD*				Group, mean ± SD*			
Characteristic	Patients (n = 25)	Controls (n = 35)	Diagnosis effect	p value	Low methylation	High methylation	Effect	p value
Age	41.6 ± 10.8	35.6 ± 13.0	$t_{58} = 1.9$	0.06	35.0 ± 41.9	41.9 ± 11.2	$t_{58} = 2.2$	0.031
Sex, female:male	23:10	21:15	$\chi^2_{58} = 0.55$	0.45	24:9	16:11	$\chi^2_{58} = 1.2$	0.27
Height, cm	169.5 ± 8.7	172.5 ± 10.2	$t_{58} = 1.2$	0.25	169.9 ± 9.7	172.9 ± 10.2	$t_{58} = 1.2$	0.25
Weight, kg	75.2 ± 17.2	70.0 ± 15.8	$t_{58} = 1.2$	0.23	68.5 ± 16.9	76.7 ± 15.1	$t_{58} = 1.9$	0.06
HAM-D score	29.3 ± 6.4	2.7 ± 3.1	$t_{58} = 19.1$	< 0.001	12.1 ± 13.9	15.9 ± 14.1	$t_{58} = 1.1$	0.29
BDI score	34.8 ± 12.5	2.4 ± 3.3	$t_{58} = 12.7$	< 0.001	13.1 ± 16.7	19.3 ± 19.6	$t_{58} = 1.3$	0.19
Age at onset, yr	25.3 ± 12.0	—	—	—	26.5 ± 6.5	24.2 ± 15.2	$t_{58} = 0.4$	0.68
Cumulative illness duration, yr	9.4 ± 9.0	—	—	—	9.8 ± 9.9	9.2 ± 8.5	$t_{58} = 0.17$	0.87
Duration of treatment, d	2251 ± 2711	—	—	—	1770 ± 2441	2693 ± 2973	$t_{58} = 0.81$	0.41
Depression without treatment, d	1906 ± 2985	—	—	—	2320 ± 3241	1527 ± 2818	$t_{58} = 0.63$	0.54
Medication (none/SSRI/dual-acting)	8/10/7	—	—	—	26/3/4	17/7/3	$\chi^2_{58} = 3.0$	0.22

BDI = Beck Depression Inventory; HAM-D = Hamilton Rating Scale for Depression; SD = standard deviation; SSRI = selective serotonin reuptakte inhibitors.
*Unless indicated otherwise.

However, participants with higher *SLC6A4* methylation had significantly lower activation than those with lower *SLC6A4* methylation in the right posterior insula and right rolandic operculum (p = 0.005, FWE-corrected) in higher order cognitive tasks when switching attention away from negative stimuli in contrast to shifting attention away from positive stimuli. Less activation was also seen in participants with higher *SLC6A4* methylation than those with lower *SLC6A4* methylation in the superior temporal lobe when shifting attention away from neutral stimuli (p = 0.029, FWE-corrected) and in the pons when shifting attention away from positive stimuli in contrast to focusing on the same emotion (p = 0.004, FWE-corrected; Fig. 1). This decrease in activation with higher methylation status was mainly seen in the patient group (Table 2).

Results were comparable when analyzing the methylation data on a linear scale. Specifically, significant linear correlations were detected between mean *SLC6A4* methylation across investigated CpG sites and BOLD responses in the left insula/inferior frontal operculum (r = 0.45, p < 0.001; Fig. 1) for processing stimuli with negative emotional content. Moreover, *SLC6A4* methylation was associated with BOLD responses in the right insula/inferior frontal operculum when switching away from negative compared with positive stimuli (r = –0.48, p < 0.001; Fig. 1) and in the pons when switching attention away from positive emotional stimuli toward the geometry instead of staying focused on the positive emotion (r = –0.44, p < 0.001). These significant correlations were also detected for the specific CpG sites 5–14, particularly 5, 6, 11 and 12, and remained significant after controlling for medication use.

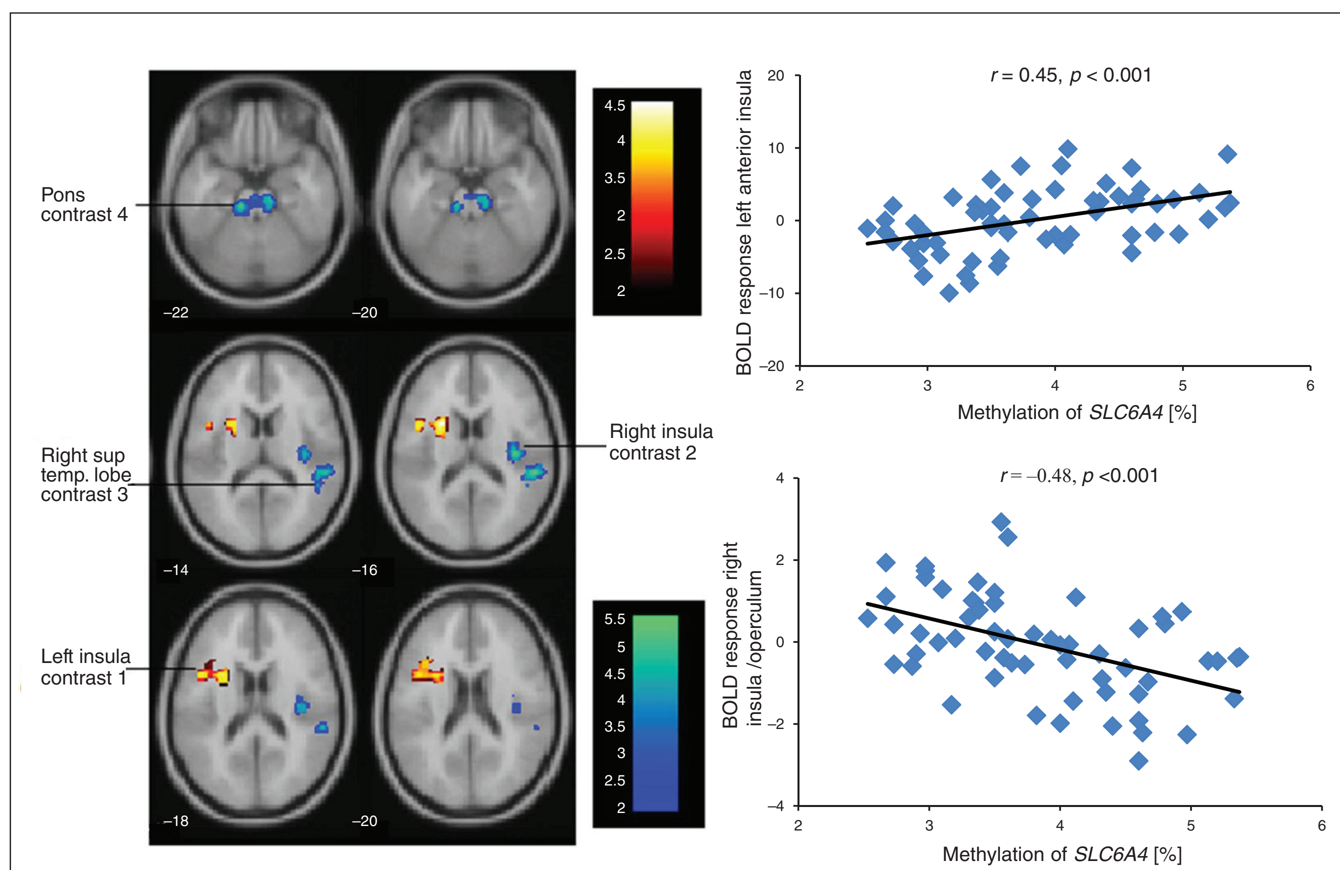

Fig. 1: (Left) Differences between participants with high versus low methylation in blood DNA measured in fMRI. Areas in red–yellow indicate higher activation in participants with high *SLC6A4* methylation compared with those with a low amount of *SLC6A4* methylation for the contrast negative stimuli versus neutral stimuli (left insula, inferior frontal operculum region, p = 0.002, family-wise error [FWE]–corrected). Areas in blue depict lower activation in participants with high levels of *SLC6A4* methylation compared with those with a low level of *SLC6A4* methylation. **(A)** the right insula (p = 0.005, FWE-corrected) when shifting attention away from negative stimuli in contrast to switching attention away from positive stimuli participants. **(B)** the right superior temporal lobe (p = 0.029) when shifting attention away from neutral in contrast to focusing on the emotion. **(C)** The pons, cerebellum (p = 0.004) region when shifting attention away from positive stimuli in contrast to focusing on the emotion. **(Right)** Association between methylation of *SLC6A4* determined by pyrosequencing (values indicate % methylation at CpG site; X axis) and brain blood oxygen–level dependent (BOLD) response (Y axis). **(Top)** During processing of negative emotional stimuli the BOLD response in the left insula/frontal inferior operculum cluster was positively associated with DNA methylation (r = 0.45, p < 0.001). **(Bottom)** When shifting attention away from negative stimuli in contrast to switching attention away from positive stimuli the right insula/inferior frontal operculum was negatively associated with methylation (r = –0.48, p < 0.001).

Diagnosis by methylation interactions

There was a significant statistical interaction effect between the methylation and diagnostic group in the hippocampus/amygdala region when viewing negative or positive picture stimuli in contrast to neutral picture stimuli (Table 3). Disentangling the interaction showed that controls with higher *SLC6A4* methylation depicted significantly more activity bilaterally in the hippocampus/amygdala region than controls with lower *SLC6A4* methylation when viewing positive stimuli in contrast to neutral stimuli. Healthy controls with higher *SLC6A4* methylation showed significantly more activation in large clusters of the left inferior frontal operculum and left fusiform gyrus that extended to the hippocampal area as well when viewing negative stimuli in contrast to neutral stimuli (Fig. 2). We detected no significant interactions between valence and methylation effects.

Group comparison

Patients with MDD showed significantly increased BOLD responses elicited by emotional stimuli and decreased BOLD responses during switching away from emotional stimuli compared with healthy controls. Regions mainly found to be overactivated in response to emotional stimuli were the right hippocampus, middle cingulate cortex, precuneus and right precentral gyrus (all $p < 0.05$, FWE-corrected). Interestingly, the BOLD response was also increased in the right insula after the presentation of positive stimuli ($p < 0.001$, uncorrected). Shifting attention away from negative stimuli compared with focusing on the same emotion elicited less BOLD response in the hippocampus in patients with MDD than controls ($p < 0.001$, uncorrected); however, the latter 2 findings did not survive correction for multiple comparisons.

Adding medication status (none, selective serotonin reuptake inhibitors [SSRIs], dual acting substances) as a covariate did not change the associations between methylation of *SLC6A4* and brain function and did not affect differences between patients and controls reported here.

Discussion

The present study showed a significant effect of the state of *SLC6A4* methylation in whole blood DNA on fMRI BOLD responses during emotional attention processing. These findings were obtained in the correlation analysis and in the ANCOVA (high v. low methylation). The BOLD responses elicited by negative emotional stimuli in the left anterior insula/frontal operculum area were found to be positively associated with methylation of the *SLC6A4* regulatory region, whereby BOLD responses in the right posterior insula, temporal lobe and pons elicited by shifting attention away from emotions were significantly negatively correlated with *SLC6A4* methylation. Interestingly, patients with MDD showed increased activation in the insula and other emotional brain regions elicited by relevant emotional stimuli and lower activity in the hippocampal area during higher-order cognitive processing compared with controls, therefore, suggesting that state of *SLC6A4* methylation and depression influence brain function in the same direction.

Interestingly, the anterior insula and the adjacent frontal operculum play a major role for emotional awareness of oneself and of others.[31–34] In line with a functional differentiation between the anterior[35] and posterior[36] insular cortex, the anterior insular cortex was associated with methylation of *SLC6A4* during emotional processing of negative stimuli, whereas the posterior insular cortex seems to be associated with methylation of *SLC6A4* when someone shifts attention

Table 2: Significant findings from the ANCOVA using age and sex as covariates corrected for the whole brain using family-wise error correction

					MNI coordinates		
Contrast	Region for maxima	*p* value*	k	*t*	*x*	*y*	*z*
Negative – neutral pictures							
High > low methylation	Left anterior insula	0.002	160	4.54	–27	8	16
	Left inferior frontal operculum			4.18	–45	2	17
	Left inferior frontal operculum			4.09	–39	17	22
Focusing on geometrics following negative pictures – focusing on geometrics following positive pictures							
High < low methylation	Right rolandic operculum / insula	0.005	50	5.71	39	–19	16
Focusing on geometrics – focusing on emotional content both following neutral pictures							
High < low methylation	Right superior temporal lobe	0.029	92	5.23	54	–34	16
Focusing on geometrics – focusing on emotional content both following positive pictures							
High < low methylation	Pons	0.004	172	4.51	–12	–28	–26

ANCOVA = analysis of covariance; FWE = family-wise error; MNI = Montreal Neurological Institute.
*FWE-corrected.

away from emotional content. In a recent meta-analysis of fMRI studies comparing patients with MDD and controls, increased activity was found in the left insula and decreased activity in the right insula,[9] which is in agreement with the difference in associations between methylation with the left and right insula cortex in the present study. Lower brain reactivity in the pontine raphe area was found to be associated with higher methylation of *SLC6A4*, and this might be in line with experimental research showing that in the pontine raphe area serotonin was associated with autoinhibitory feedback to serotonin neurons.[37] The superior temporal lobe is involved in the perception of emotional stimuli,[38] and this function might explain why it associated with methylation of *SLC6A4*.

Our study, using peripheral DNA methylation markers of a regulatory region of the serotonin transporter, expands on the relevance of serotonergic neurotransmission and the serotonin transporter gene for processing of relevant emotional information. Modulation of neuronal response in the insula by administration of selective SSRIs has been shown very consistently in fMRI studies.[39–42] Moreover, carriers of the short allele of the functional polymorphism in the promoter region of *SLC6A4* exhibited greater amygdala BOLD reactivity in response to fearful stimuli than individuals homozygous for the long allele of this polymorphism.[43] A recent meta-analysis confirmed the association between 5-HTTLPR and amygdala activation, but also indicated that this association is weaker than originally thought.[44] Thus, methylation of *SLC6A4* seems be an important additional factor that accounts for variance in fMRI results.

Although it is impossible to determine the state of methylation of *SLC6A4* in the respective brain regions in living individuals, the striking correlation between DNA methylation in the periphery and exquisite anatomic features of brain reactivity support the hypothesis that peripheral DNA methylation of *SLC6A4* shares functionally relevant information about these brain regions. Although the common wisdom in the field has been that brain-relevant DNA methylation differences should be measured in the brain only, our study provides further support to the hypothesis that particular DNA methylation states that are relevant to brain function are detectable in the periphery. This has important implications for future use of noninvasive DNA methylation markers in diagnosis and risk prediction in mental health.

Interestingly, the present study also showed interactive effects between methylation of *SLC6A4* in the periphery and health state on brain reactivity. Within the amygdala, a primary region for emotion recognition,[45] and the hippocampus, BOLD responses were increased in healthy controls with a high degree of *SLC6A4* methylation compared to controls with low *SLC6A4* methylation in response to positive stimuli. Moreover, healthy participants with higher methylation of *SLC6A4* also depicted enhanced BOLD responses in the hippocampal area, particularly in the left inferior operculum and left fusiform gyrus, elicited by negative stimuli compared with those with low *SLC6A4* methylation. This finding might indicate a stronger use of cognitive control strategies in healthy participants with a high degree of *SLC6A4* methylation. Since epigenetic variations are known to be affected by childhood maltreatment, they might be involved in mediating the impact of childhood maltreatment on emotional reactivity later in life. These findings might therefore suggest that this healthy group learned resilience strategies to focus on positive emotions and regulate negative emotions by involving cognitive control regions. Studies with larger samples that can further investigate the interactive effects of childhood maltreatment, methylation and diagnosis are required to address this question in detail.

Also noteworthy was the finding that patients with MDD judged positive or neutral stimuli as not being positive or neutral, respectively, significantly more often than controls, which is in line with the known negative processing bias in patients with MDD.[46] Moreover, patients with MDD responded significantly slower and depicted more BOLD reactivity after emotional stimuli and less reactivity after higher-order cognitive processing than healthy controls, which is in line with the findings of previous studies.[47] In confirmation of previous research, BOLD response elicited by relevant emotional stimuli was increased in the hippocampus, precuneus, cortex cinguli, right insula, dorsomedial and dorsolateral prefrontal cortex in patients with MDD compared with healthy controls.

Table 3: Significant findings from the ANCOVA using age and sex as covariates corrected for the whole brain using family-wise error correction

					MNI coordinates		
Contrast	Region for maxima	*p* value*	k	*t*	*x*	*y*	*z*
Negative – neutral pictures							
Interaction (diagnosis × methylation)	Hippocampus	< 0.001	266	4.24	15 –6	–7 –13	–8 –14
High methylation > low methylation in controls	Left frontal inferior operculum	< 0.001	296	4.79	–45	5	16
	Left fusiform gyrus (extending to hippocampal area)	< 0.001	630	4.68	–24	–37	–17
Positive – neutral picture							
Interaction (diagnosis × methylation)	Right hippocampus	0.06	52	4.47	15	–7	–11
High methylation > low methylation in controls	Right hippocampus	0.012	117	5.24	15	–10	–14
	Left amygdala/hippocampus	0.015	111	4.49	–21	–1	–11

FWE = family-wise error; MNI = Montreal Neurological Institute.
*FWE-corrected.

Limitations

A strength of the present study was that the investigated *SLC6A4* region was based on previous work conducted in an independent sample. The interpretation of the present study, however, rests upon the following limitations. First, because it is impossible to study DNA methylation directly in the living brain, peripheral cells were used to assess methylation. Nevertheless, there is emerging evidence that methylation patterns of genes in the periphery are informative for the understanding of behavioural or brain functions,[19,48] and the present study contributes to this evidence. Second, since this and most of the other studies on this topic are correlational, we cannot draw any conclusion about cause and effect. Longitudinal studies that will demonstrate a temporal relationship between *SLC6A4* methylation and the emergence of alterations in brain function described here are required. Third, the fMRI BOLD response differences reported here might just be an epiphenomenon related to the serotonergic system and might not help us to better understand the pathophysiology of depression, because methylation of *SLC6A4* was not related to depression. Although this was not a primary aim of the present study, we tested for group differences in *SLC6A4* methylation and did not find significant differences between patients with MDD and healthy controls. However, because methylation of *SLC6A4* was found to be associated with childhood adversity in previous research[15–18] and confirmed in the present study, there seems to be an important feature of psychopathology that needs to be further investigated. While the sample was large enough to investigate effects of methylation on brain function, it was too small to draw reliable conclusions about genetic effects on behavioural measures like clinical symptoms. In addition, other factors, including resilience factors, are important in the risk for depression. We tentatively found a strategy of the brain to regulate negative emotional content in healthy participants with a high degree of *SLC6A4* methylation that may decrease the risk for depression. Furthermore, other genetic factors must to be taken into account. For example, the serotonin transporter genotype was not included in the present analysis, because our sample size statistically allowed only for investigating methylation and diagnosis as factors. Future larger studies should take environmental, epigenetic and genetic factors into account. Fourth, it also has to be considered that childhood maltreatment assessed retrospectively only covers the later childhood; earlier development periods, such as fetal periods, early childhood and transgenic aspects, are not covered. Another limitation was treatment with antidepressants in some patients. Adding medication status to the analysis did not change the statistical results; however, this should be replicated in an independent sample that also includes medication-free patients.

Conclusion

The present study provides further validation for these particular *SLC6A4* DNA methylation states as peripheral markers of brain functional states. Furthermore, the findings suggest that healthy individuals with high *SLC6A4* methylation might use resilience strategies to manage negative affect and to react and be aware emotionally of positive situations effectively.

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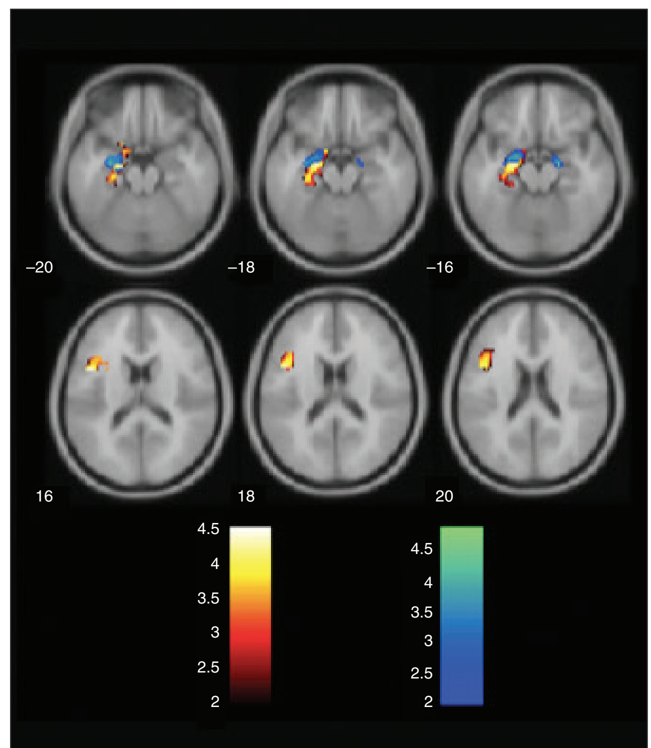

Fig. 2: Healthy participants with higher *SLC6A4* methylation values depicted significantly more activity when viewing stimuli with emotional content in contrast to neutral stimuli than participants with low levels of *SLC6A4* methylation. They showed more activity in limbic regions like the hippocampus and amygdala when seeing positive stimuli (blue), but showed more activity in cognitive control regions like the inferior frontal operculum (red–yellow) in addition to the left hippocampus (red–yellow) when seeing negative stimuli compared with neutral stimuli.

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