OPEN

Citation: Transl Psychiatry (2017) 7, e1167; doi:10.1038/tp.2017.133

www.nature.com/tp

ORIGINAL ARTICLE

Serotonin transporter gene promoter methylation status correlates with *in vivo* prefrontal 5-HTT availability and reward function in human obesity

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A polymorphism in the promoter region of the human serotonin transporter (5-HTT)-coding *SLC6A4* gene (5-HTTLPR) has been implicated in moderating susceptibility to stress-related psychopathology and to possess regulatory functions on human *in vivo* 5-HTT availability. However, data on a direct relation between 5-HTTLPR and *in vivo* 5-HTT availability have been inconsistent. Additional factors such as epigenetic modifications of 5-HTTLPR might contribute to this association. This is of particular interest in the context of obesity, as an association with 5-HTTLPR hypermethylation has previously been reported. Here, we tested the hypothesis that methylation rates of 14 cytosine–phosphate–guanine (CpG) 5-HTTLPR loci, *in vivo* central 5-HTT availability as measured with [^{11}C]DASB positron emission tomography (PET) and body mass index (BMI) are related in a group of 30 obese (age: 36 ± 10 years, BMI > 35 kg/m^2) and 14 normal-weight controls (age 36 ± 7 years, BMI < 25 kg/m^2). No significant association between 5-HTTLPR methylation and BMI overall was found. However, site-specific elevations in 5-HTTLPR methylation rates were significantly associated with lower 5-HTT availability in regions of the prefrontal cortex (PFC) specifically within the obese group when analyzed in isolation. This association was independent of functional 5-HTTLPR allelic variation. In addition, negative correlative data showed that CpG10-associated 5-HTT availability determines levels of reward sensitivity in obesity. Together, our findings suggest that epigenetic mechanisms rather than 5-HTTLPR alone influence *in vivo* 5-HTT availability, predominantly in regions having a critical role in reward processing, and this might have an impact on the progression of the obese phenotype.

Translational Psychiatry (2017) **7,** e1167; doi:10.1038/tp.2017.133; published online 4 July 2017

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) transporter (5-HTT) has a crucial role in regulating emotional and reward processing.[1–3] Changes in 5-HTT availability and thus synaptic serotonin levels have been associated with stress-related diseases such as mental and metabolic disorders including obesity.[4–10]

Specifically, obesity has been associated with reduced subcortical (caudate–putamen–thalamus) 5-HTT availability[11] and increased 5-HT$_4$ receptor availability in the nucleus accumbens and ventral pallidum,[12] regions that are intimately involved in reward processing. These changes are possibly compensatory in nature because of reduced brain serotonin levels,[13] which is well known to promote a negative energy balance through suppression of appetite. The precise mechanisms however that lead to changes in 5-HTT availability *in vivo* remain unclear.

At the genetic level, a 43-bp insertion/deletion polymorphism in the promoter region upstream of the 5-HTT-coding sequence (5-HTTLPR) of the *SLC6A4* gene has been shown to result in a short (S) and a long (L) allelic variant that alters *SLC6A4* transcription and the magnitude of 5-HTT function.[1,14] As a result, carriers of the homozygous L-allele variant exhibit elevated concentrations of 5-HTT mRNA and express nearly twofold greater 5-HT re-uptake in contrast to S-allele carriers. Consequently, the existence of S-allele lowers the transcriptional activity of 5-HTTLPR, leading to diminished 5-HTT expression and 5-HT reuptake. However, positron emission tomography (PET) studies using the highly 5-HTT-selective radiotracer [^{11}C]-3-amino-4-(2-dimethylamino-methylphenylsulfanyl)-benzonitrile (DASB) have produced inconclusive results on 5-HTTLPR genotype and *in vivo* 5-HTT availability in the human brain.[15–18]

To further strengthen our understanding of how DNA sequence-based genetic variation shapes individual differences in human brain 5-HTT function, behavioral traits and related risk for psychopathology, non-sequence-based epigenetic variation, that is, DNA methylation of 5-HTTLPR, need to be considered, but as yet remain relatively unexplored. DNA methylation mainly occurs at DNA sections where cytosine is succeeded by guanine (cytosine–phosphate–guanine (CpG) dinucleotides). Site-specific changes typically involve DNA methyltransferases transferring a methyl group to position five of the cytosine ring of most CpG dinucleotides in the mammalian genome without affecting the

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Received 28 November 2016; revised 8 April 2017; accepted 9 May 2017

DNA sequence.[19,20] Recent data have shown a potential link between epigenetic variation, for example, methylation in CpG-rich regions of 5-HTTLPR and behavior.[21–24] Explorative studies also revealed that 5-HTTLPR methylation patterns of different CpG loci are a functionally relevant biomarker of reduced *in vivo* 5-HTT expression (assessed from peripheral blood cells)[25–27] associated with behavioral outcomes.[24,28] Specifically, increased 5-HTTLPR methylation predicted an increased threat-related amygdala reactivity *in vivo* and decreased mRNA expression in postmortem amygdala tissue from a third independent cohort (to gain further mechanistic insight into the *in vivo* findings)[29] functionally coupled with key nodes of the salience network.[30]

Furthermore, epigenetic variations in 5-HTTLPR are increasingly being discussed as an important risk factor for obesity.[21] In a monozygotic twin study covering 84 twin pairs, Zhao *et al*. found *SLC6A4* promoter hypermethylation significantly associated with an increased prevalence of obesity.[21] In addition, 5-HTTLPR DNA methylation in the prefrontal cortex (PFC) has been described to be a highly dynamic process modified by genetic variance and regulating gene transcription due to fast changes of DNA methylation patterns during the prenatal period that slows down after birth and continues to slow down with aging.[31] In the PFC, serotonin represents a major modulator of its function.[32] The PFC exhibits a dense expression of serotonergic receptors and represents a region of serotonergic innervation.[32] Here, 5-HT_{1A}, 5-HT_{2A} and 5-HT_{3A} receptors are selectively expressed in distinct populations of pyramidal neurons and inhibitory interneurons.[32] This is of particular interest, as the PFC has also been linked with obesity as shown by resting-state and task-related functional magnetic resonance imaging studies.[33–35] Thus, a lesser degree of centrality in the left middle frontal gyrus has been demonstrated, suggesting the important role of the middle frontal gyrus in the pathophysiology of obesity.[33]

5-HT has also been discussed as a fundamental mediator of emotional, motivational and cognitive aspects of reward representation.[36] For instance, Nakamura *et al.* demonstrated in non-human primates that, unlike dopamine-containing neurons of the substantia nigra, serotonin-containing neurons of the dorsal raphe nucleus encode the value of a received fruit juice reward.[37] Moreover, the study of Choi *et al*. revealed interactions between reward and threat processing in regions of human PFC, that is, the middle frontal gyrus, but also in other distinct brain regions,[38] that is, the nucleus accumbens, a key neural substrate in the reward system since its involvement in natural (that is, food) reinforcement.[39]

No studies have been conducted so far comparing 5-HTTLPR methylation and *in vivo* 5-HTT availability in the PFC, in particular, in human obesity in the context of reward processing. The primary aim of the present study was, therefore, to investigate whether 5-HTTLPR methylation has an impact on *in vivo* 5-HTT availability in obese and normal-weight individuals. As methylation rates are assumed to be associated with low transcription rates,[19,40,41] we hypothesized that elevated methylation rates of 5-HTTLPR result in lower *in vivo* 5-HTT availability. Second, we investigated whether there is an impact of CpG-associated 5-HTT availability on measures of behavioral control in 5-HT-dependent regulatory areas of the PFC.[42] To assess reward function, we used the behavioral approach system (BAS) reward scale as our own previous study data indicated that 5-HTT availability is associated with levels of BAS reward determined by questionnaire score scales in the PFC. Finally, to account for covariates, we studied whether body mass index (BMI) and age have an effect on a possible association between CpG and 5-HTT availability. 5-HTTLPR methylation rates were assessed from peripheral blood cells as a proxy for central *in vivo* 5-HTT.[43–45]

MATERIALS AND METHODS

Study population

The study cohort consisted of 44 non-depressed volunteers (Caucasians) covering an age range of 21–59 years with a mean age of 36 ± 9 years and a BMI range of 19–54 kg/m^2. This included 14 study participants with a BMI < 27 kg/m^2 (mean age 36 ± 7 years, 9 female) and 30 obese participants with a BMI > 35 kg/m^2 (mean age 36 ± 10 years, 20 female) as previously described.[46] All participants gave their written informed consent.

Study participants were clinically examined by a physician and assessed for the mental illness via structured interview by a psychiatrist. Exclusion criteria comprised present or past alcohol abuse and/or drug abuse, current or past neurological or psychiatric diseases, epilepsy, migraine, epileptic seizures in their previous medical history (or in the family history), neurosurgical interventions in the past, pregnancy or other medical conditions as well as drugs that may alter or influence brain function (for example, antidepressants). Study participants were running an Obesity program in the Ambulance of the IFB AdiposityDiseases in Leipzig, Germany, which includes metabolic status before selecting patients for studies. They were investigated regarding type I or insulin-dependent type II diabetes. Patients with diabetes requiring insulin therapy as well as those with poorly controlled diabetes (HbA_{1c} > 7%) were excluded from the study. Exclusion criteria also covered contraindications for magnetic resonance imaging (for example, implanted ferromagnetic devices, claustrophobia), which was performed to exclude structural lesions such as traumatic brain injuries and stroke. Magnetic resonance imaging included sequences for anatomical data co-registration (3 T, Siemens, Erlangen, Germany; T1-weighted 3D magnetization prepared rapid gradient echo; time of repetition: 2300 ms, time of echo: 2.98 ms, 176 slices, field of view: 256 × 240 mm, voxel size: 1 × 1 × 1 mm).

On the day of PET imaging, the extent of depressive symptoms was rated using the Beck Depression Inventory.[47] On the same day prior to PET scanning, typical laboratory parameters, for example, glucose (mg dl^{-1}), insulin (mg dl^{-1}) and Hba_{1c} (mmol l^{-1}), were assessed and peripheral blood samples for genotyping were collected and immediately refrigerated at − 80 °C.

The study was conducted in accordance with the Helsinki Declaration and approved by the ethics committee of the Medical Faculty of the University of Leipzig registered under the number 206-10-08032010 and the German Bundesamt für Strahlenschutz/Federal Office for Radiation Protection (number Z5-22461-2-2011-002). The study was registered at the European clinical trial database (EudraCT 2012-000568-32) and the German Clinical Trials Register (DRKS).

The behavioral inhibition system/behavioral activation system scales

In addition, the sensitivity to reward and punishment as based on the behavioral inhibition system/behavioral activation system (BIS/BAS) scales[48] was measured as a relevant personality factor in the context of BMI.[49] This self-report questionnaire consists of 20 items designed to assess the responsiveness of BAS and BIS personality characteristics. The 7-item BIS scale measures the reactivity of the aversive motivational system, whereas the13-item BAS scale measures reactivity of the appetitive motivational system. The BAS scale can be divided into three subscales: drive, fun-seeking and reward.

PET image acquisition

The 5-HTT selective, carbon-11-labeled radiotracer was applied as previously described.[7,46] After 10 min transmission scan from three ^{68}Ga sources for attenuation correction and intravenous bolus injection (90 s) of 484 ± 10 MBq [^{11}C]DASB a dynamic PET scan was performed to collect emission data by using an ECAT EXACT HR+ scanner (Siemens; intrinsic resolution at the center 4.3 mm (full width at half maximum, FWHM), axial resolution: 5–6 mm, field of view: 15.5 cm, 3–4 mm FWHM) in a 3D mode over 90 min (23 frames: 4 × 0.25, 4 × 1, 5 × 2, 5 × 5, 5 × 10 min). Data were iteratively reconstructed (10 iterations, 16 subsets) in transverse image series (63 slices, 128 × 128 matrix, voxel size 2.6 × 2.6 × 2.4 mm) with a Hann filter (cutoff 4.9 mm).

Imaging data processing

Individual magnetic resonance imaging data sets of the participants were spatially reoriented onto a standard brain data set, defined by the anterior

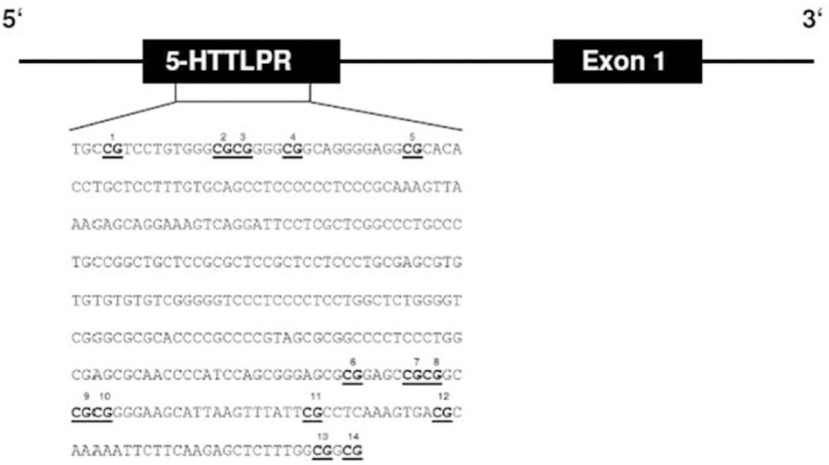

Figure 1. Exposure of SLC6A4 gene promoter region for bisulfite sequencing analysis. Analyzed cytosine–phosphate–guanine (CpG) sites are bolded and underlined. Assay design was based on the Origene ID NM_001045.

and posterior commissure (AC-PC), lie on a straight horizontal line similar to AC-PC orientation—using PMOD software (version 3.4, PMOD Technologies, Zurich, Switzerland). 5-HTT binding potential (BP_{ND}) maps were generated by using the multilinear reference tissue model (MRTM2).[42] The raphe dorsalis was used as receptor-rich region. The cerebellar cortex (without vermis) referred as reference region.[50] The estimation of k2 occurred individually.

Genotyping of 5-HTTLPR

To determine the bi-allelic status, genomic DNA was extracted from 1 ml of a 5–10 ml sample of peripheral blood with ethylenediaminetetraacetic acid as anticoagulant. Isolation steps were performed using pegGold DNA Mini kit (pegLab, Erlangen, Germany) according to the manufacturer's instructions. 5-HTTLPR gene polymorphism study was performed with standardized polymerase chain reaction amplification procedure.[51] Utilized primer sequences were 5'-GAGGGACTGAGCTGGACAAC-3' and 5'-GCAGCA GACAACTGTGTTCATC-3', yielding to a product length of ~620 bp for the L-allele, and 583 bp for the S-allele. Used primers were purchased from Invitrogen (Paisley, UK).

DNA methylation analysis by quantitative bisulfite pyrosequencing

Methylation rates were determined including 14 CpG-loci[21] by using the PyroMark CpG-assays (Qiagen, Hilden, Germany; see Figure 1) based on the Origene ID NM_001045. The assays were designed to capture the CpG sites in the promoter region upstream of the transcriptional start site and are targeted to the antisense sequence of *SLC6A4*.[51] Genomic DNA was extracted by DNeasy Blood & Tissue Kit (Qiagen) from peripheral blood leukocytes. Bisulfite conversion was performed by using 500 ng genomic DNA using Qiagen Epitect Bisulfite Kit according to the manufacturer's protocol. Pyrosequencing was executed using pyrosequencing instrument technology (Qiagen). Pyrogram analyses occurred via PyroMark Q24 software (version 2.0.6, Qiagen). Each subject sample was independently replicated on different plates. At every CpG locus, the mean percentage (%) of methylation was calculated.

Data analysis

A voxel-wise statistical analysis of BP_{ND} maps was performed using SPM8 (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurobiology, UK) implemented in Matlab 7.13 (The MathWorks, Natick, MA, USA). Motion correction was performed as implemented in SPM. The mean images (frames 1–13, 0–15 min) were used to determine the spatially normalization matrix on the SPM8-integrated PET template and subsequently applied to the BP_{ND} maps. Brain regions were denoted in accordance to the nomenclature by the LONI Probabilistic Brain Atlas (LPBA40).[52] Here, only regions of the PFC by using a prefrontal mask were considered (that is, the left inferior frontal gyrus, the right middle frontal gyrus, the left middle frontal gyrus and the right inferior frontal gyrus (Supplementary Table S1)). Smoothing was performed with an 8 mm FWHM Gaussian filter. Group differences in BP_{ND} maps between LL and S+Carriers were tested voxelwise in SPM using a two-sampled *t*-test with methylation rate as nuisance variable and predefined prefrontal mask as explicit mask. A one-sample *t*-test was used to test for correlation of parametric BP_{ND} images and methylation rate throughout the predefined prefrontal mask in SPM. We report effects for clusters of voxels exceeding a peak level threshold of $P < 0.001$ (uncorrected) and a cluster size threshold of $P < 0.05$, family-wise error-corrected. For the voxelwise correlation analysis, the first eigenvariate of significant clusters was extracted and correlated with BAS questionnaire scores. The first eigenvariate represents the weighted mean of BP_{ND} in clusters defined by their significant correlation to the CpG locus methylation rate.

Statistical analysis

In SPSS (version 21, SPSS, IBM, Chicago, IL, USA), all obtained data were tested for normal distribution through application of Shapiro–Wilk test. Unpaired *t*-test was performed in the analysis of differences observed between groups (that is, comparison on CpG loci methylation rates between 5-HTTLPR variants, Table 1). To test for correlations between the 5-HTT BP_{ND} and other investigated parameters (for example, BAS reward, BAS fun, BAS drive, BIS and BMI), two-sided Pearson's correlation analysis was applied. Bonferroni correction was carried out to correct for multiple comparisons.

RESULTS

Sample characteristics

Application of Shapiro–Wilk test revealed that all obtained data were normally distributed ($P \geqslant 0.05$).

Effects of 5-HTTLPR genotypes on demographics, neuropsychological scores and methylation rates

Subjective parameters stratified for 5-HTTLPR genotypes of all study participants demonstrated similar data distribution: homozygous LL and S+ carriers did neither significantly differ in demographics, neuropsychological scores nor in their methylation rates in the distinct CpG loci (Table 1). Voxel-wise SPM genotype group comparison (LL versus S+Carriers) with CpG 10 loci as nuisance covariate revealed no significant differences, neither in obese nor in normal-weight individuals (data not shown).

Effects of methylation on BP_{ND} in obese versus normal-weight individuals

In SPM analysis, we found 5-HTTLPR methylation rates in locus CpG10 to be significantly negatively correlated with BP_{ND} across

clusters of PFC: left inferior frontal gyrus, right middle frontal gyrus, left middle frontal gyrus and right inferior frontal gyrus (Figures 2 and 3 and Supplementary Information S1). The results were found only in obese but not in normal-weight individuals. All other investigated CpG loci showed no significant correlations with BP_{ND} in clusters of PFC in obese or normal-weight individuals.

Table 1. Characteristics of participants and comparison on CpG loci methylation rates between 5-HTTLPR variants ($n = 44$)

	LL	*S+*	P-*value*
Characteristics of participants			
n	21	23 (2)	
Age (years)	35.3 ± 8.8	37.4 ± 9.8	0.47[a]
Weight (kg)	105.1 ± 28.1	104.6 ± 32.9	0.96[a]
BMI (kg/m^2)	35.5 ± 8.8	35.1 ± 10.9	0.88[a]
BDI	7.4 ± 6.5	7.5 ± 7.2	0.96[a]
Sex (female/male)	16/7	15/6	1[b]
Activity applied (MBq)	483.0 ± 12.5	487.2 ± 7.6	0.35[a]
BAS fun	11.9 ± 2.3	11.1 ± 1.4	0.19[a]
BAS reward	17.1 ± 2.2	16.1 ± 1.9	0.11[a]
BAS drive	12.9 ± 1.8	12.6 ± 2.0	0.53[a]
BIS	20.1 ± 3.1	18.0 ± 3.6	0.05[a]
CpG loci methylation rates			
CpG1	2.4 ± 0.3	2.4 ± 0.3	0.62[a]
CpG2	3.5 ± 0.5	3.5 ± 0.6	0.98[a]
CpG3	1.6 ± 0.3	1.6 ± 0.2	0.84[a]
CpG4	2.0 ± 0.4	1.8 ± 0.3	0.08[a]
CpG5	3.0 ± 0.6	2.7 ± 0.5	0.18[a]
CpG6	2.9 ± 1.0	2.8 ± 0.6	0.81[a]
CpG7	4.7 ± 0.7	4.7 ± 0.4	0.80[a]
CpG8	4.6 ± 1.0	4.7 ± 0.5	0.77[a]
CpG9	3.3 ± 0.7	3.4 ± 0.4	0.57[a]
CpG10	2.2 ± 0.5	2.1 ± 0.4	0.45[a]
CpG11	3.3 ± 0.5	2.9 ± 0.4	0.03[c]
CpG12	2.7 ± 0.6	2.6 ± 0.3	0.81[a]
CpG13	2.4 ± 0.5	2.6 ± 0.4	0.19[a]
CpG14	7.0 ± 1.5	6.8 ± 1.3	0.67[a]

Abbreviations: BAS, behavioral approach system; BDI, Beck Depression Inventory; BIS, behavioral inhibition system; BMI, body mass index; CpG, cytosine–phosphate–guanine; LL, homozygous L carriers; S+, homozygous and heterozygous S carriers. Allele distribution was in Hardy–Weinberg equilibrium (Pearson X^2-test). The number in brackets in S+ indicates the number of homozygous S-carriers. Values represent mean ± s.d. [a]Student's *t*-test . [b]Fisher's exact test . [c]Did not survive correction for multiple testing.

Effects of BP_{ND}-associated CpG10 methylation rate on reward sensitivity

We investigated whether BP_{ND}-associated CpG10 methylation rate correlates with reward as well as covariates, that is, BMI and age by application of two-sided Pearson's correlation analysis. As presented in Figure 2 and Table 2, BP_{ND} in PFC clusters associated to methylation of locus CpG10 correlated negatively with BAS reward score ($r = -0.64$, $P = 0.001$) in the obese cohort. However, no significant correlations were found between BP_{ND} in PFC clusters associated to methylation of locus CpG10 and other investigated covariates, that is, age, BMI, BAS Fun Seeking, BAS Drive as well as BIS (Table 2).

DISCUSSION

The present study demonstrated that *in vivo* 5-HTT availability in the PFC represented by the left inferior frontal gyrus, the right middle frontal gyrus, the left middle frontal gyrus and the right inferior frontal gyrus negatively associated with the rate of methylation in locus CpG10 of the 5-HTT gene promoter. These findings were observed in obese but not in normal-weight individuals.

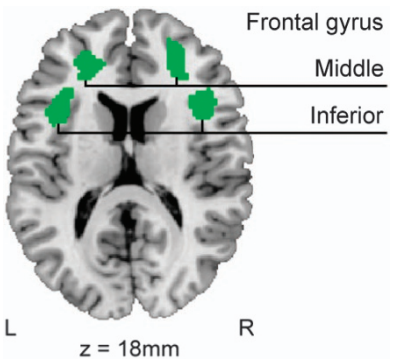

Figure 3. SPM8 projections superimposed on representative MRI indicating regions of the PFC[52] with significant negative correlation between *in vivo* BP_{ND} and methylation of locus CpG10 ($P < 0.001$ uncorrected on peak level and $P < 0.05$ FWE on cluster level, $n = 30$). For corresponding Montreal Neurological Institute coordinates see Supplementary Table 1. BP_{ND}, binding potential; CpG, cytosine–phosphate–guanine; FWE, family-wise error; MRI, magnetic resonance imaging; PFC, prefrontal cortex.

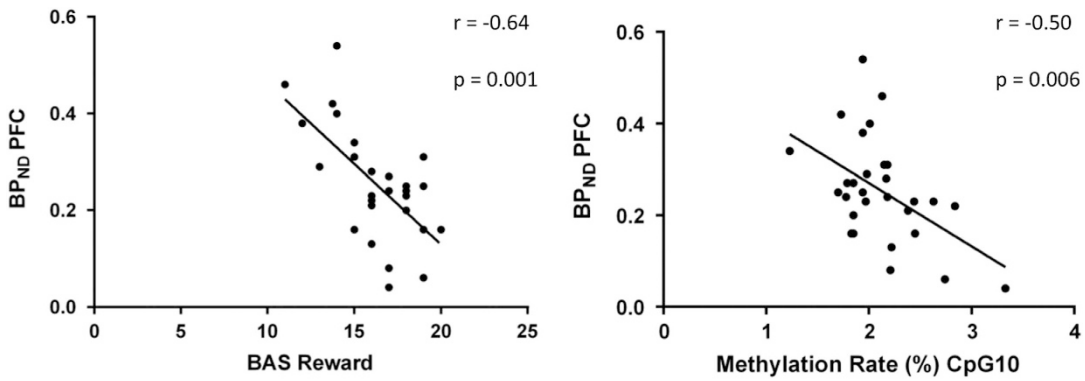

Figure 2. Significant correlations between the mean binding potential (BP_{ND}) defined by CpG10 methylation rates (unitless, *y* axis) and parameters such as: left panel: BAS reward and right panel: methylation rate measured in percent (%) of obese individuals ($n = 30$) in the prefrontal cortex (PFC). *r*, Pearson product–moment correlation coefficient; $P < 0.05$ (significant). BAS, behavioral approach system; CpG, CpG, cytosine–phosphate–guanine.

Table 2. Correlations between BP_{ND} in PFC clusters associated to methylation of locus CpG10 and other parameters such as age, BMI, BAS reward, BAS fun, BAS drive and BIS in the prefrontal cortex of obese individuals only ($n = 30$)

	CpG10	
	R	P-*value*
Age	−0.29	0.12
BMI	−0.25	0.19
BAS reward	−0.64	**0.001**
BAS fun	−0.07	0.72
BAS drive	−0.34	0.08
BIS	−0.02	0.93

Abbreviations: BAS, behavioral approach system; BIS, behavioral inhibition system; BMI, body mass index; BP_{ND}, binding potential; CpG, cytosine–phosphate–guanine; PFC, prefrontal cortex. r = Pearson product–moment correlation coefficient; bold values indicate $P < 0.05$.

The present study was intended to detect changes in the *in vivo* 5-HTT availability in a cohort of highly obese participants and in non-obese cohorts. We tried to avoid covariates in a small sample size as reported in our previous study.[46] Participants were investigated regarding metabolic status in order to exclude those with type I or insulin-dependent type II diabetes or with poorly controlled diabetes in general (HbA1c >7%). Thus, we could avoid the possibility that the studied relationship between 5-HTTLPR methylation rates may be even higher correlated with the 5-HTT availability than in the presently studied group.

Association of epigenetic modification near the 5-HTT-coding gene might explain the only modest effect size of the 5-HTTLPR genotype and inconsistent reports on allelic variation in 5-HTT function.[53] We were also not able to confirm a significant interaction between genotypic variance within 5-HTTLPR and *in vivo* 5-HTT availability[46] in both groups, normal-weight and obese individuals.

The process of DNA methylation is known to result in stable gene repression.[19,40] Our finding of elevated DNA methylation rates within 5-HTTLPR together with lower *in vivo* 5-HTT availability would be in line with the specific molecular genetic effects mediated by DNA methylation.[19]

DNA methylation is also determined by genetic variation.[54,55] Accordingly, the epigenetic effects in our study seem to be independent of (and stronger than) the effects of genotypic variance of 5-HTTLPR.[29,56]

In contrast to the study of Zhao *et al*. that showed that methylation rate of 5-HTTLPR is positively correlated with BMI,[21] we were not able to demonstrate a significant correlation between 5-HTTLPR methylation and BMI. This could be explained by the fact that this relation follows a nonlinear curve with significant association only in the highly obese. Similar nonlinear or quadratic relationships have been described for other biological markers such as the central dopamine D2 receptor availability[57] or for the central 5-HTT together with 5-HT_{2A} receptors[58] in human obesity. Instead, we found a negative relationship between methylation rates of 5-HTTLPR, *in vivo* 5-HTT availability in the PFC and the BAS reward subscale as a measure for the sensitivity to reward. This could fit in a model, as it has been proposed for the association between BMI and BAS reward with a quadratic curve indicating high reward sensitivity at moderate-to-high BMI levels.[57]

The findings of our analysis might support a link between the neurobehavioral effects of changes in modifications of the 5-HTTLPR and an enhanced sensitivity to motivationally and/or emotionally relevant environmental stimuli predicting reward. An important modulatory role in reward processing for 5-HTT was found in knockout mice in which disruptions of the development of the reward system[59] and interference with reward-based learning were found.[60] For instance, it has been shown that 5-HTTLPR/rs25531 genotype, an A/G single-nucleotide polymorphism (SNP) within the 5-HTTLPR that have been known to have a differential impact on L allele function, influences reward responsiveness.[61] S-allele carriers showed larger stress-related reduction in reward responsiveness relative to L-allele carriers.[61] Yet, we did not find a significant interaction between genotypic variance of 5-HTTLPR (without investigating o rs25531) in the PFC and BAS reward scores. Given the significant negative correlation between CpG methylation and *in vivo* 5-HTT availability with BAS reward scores, this result furthermore suggests that epigenetic effects of site-specific (that is, locus CpG10) DNA methylation influence behavioral control in regions of PFC. We assume that environmental exposure to rewarding or punishing stimuli and respective behavioral responses will thus have a greater association with methylation of 5-HTTLPR as influenced by genotypic variance.

In search of arguments for the notable outliers in the significant Pearson's correlations between the mean BP_{ND} defined by CpG10 methylation rates and BAS reward, it must be mentioned that the numbers of the included obese individuals were modest, which makes the analysis vulnerable to outliers. Therefore, we cannot exclude that these outliers significantly influence the investigated parameters such as reward.

In the present study we only focused on the 5-HTT availability in the PFC. This does not exclude any correlation between 5-HTTLPR methylation and 5-HTTLPR availability in regions outside the PFC. In that manner, we did not perform an unbiased whole-brain analysis since our own previous study showed that 5-HTT availability determines levels of BAS reward in the PFC such as the orbitofrontal cortex (unpublished). Thus, we are also not able to state whether the 5-HTTLPR methylation status varies globally or locally across different brain regions[62,63] and whether this indicates region-specific functional specialization.[63] It is possible that this region-specific functional specialization involves the distinctive expression of transcription factors that regulate 5-HTT transcription.[64] For instance, mouse midbrain serotonergic neurons selectively express the transcription factor *Pet1,* which is required for normal *SLC6A4* transcription in this region.[64] It might be speculated that human PFC neurons selectively express a transcription factor that normally increases 5-HTT expression and that is competitively displaced by CpG 10 methylation of the 5-HTT promoter.

Taken together, our finding of higher site-specific methylation of 5-HTTLPR seems to predict decreased 5-HTT availability and, consequently, a reduction of regional 5-HT re-uptake capacity in PFC[1,29] would be in agreement with previous literature,[1,29,56] and may contribute to an altered cognitive and emotional function in obesity. However, we cannot conclude from these results that epigenetic changes associated with molecular changes in the brain and behavior predisposes obesity since we could neither find a specific link of CpG-associated 5-HTT availability with BMI nor have we directly measured gene expression in our study.

LIMITATIONS

Our study has several limitations. First, we investigated peripheral blood leukocytes, which are commonly employed in *SLC6A4* methylation and allelic variation studies on the basis that peripheral patterns reflect central processes.[65,66] Prior work has provided a significant correlation between blood and brain methylomes,[43] and the 5-HTT promoter methylation level measured in peripheral blood cells has been demonstrated to be associated with central *in vivo* 5-HT synthesis as detected by

dedicated PET.[45] Moreover, studies investigating a comparison between both platelet and neuronal 5-HTT regulation are rare and inconsistent results exist concerning functional activity of 5-HTT. Second, the sample size of our study, in particular of normal-weight controls (although carefully matched for any variables), was modest: suitable for a PET study, but with limited statistical power for genetic analysis. Furthermore, we did not investigate the 5-HTTLPR/rs25531 genotype, an A/G SNP within the 5-HTTLPR to estimate the influence of the rs25531 SNP on the 5-HTTLPR function. Investigating this genotype would result in a subdivision of our modest sample size, with the consequence of limited statistical power.

As this was a pilot study to fuel new hypotheses on an interaction between epigenetics and a distinct molecular target, that is, the 5-HTT availability *in vivo*, larger sample sizes are warranted to identify the main effects of 5-HTT genotype and its modification on *in vivo* 5-HTT function in psychiatry and/or metabolic research. Moreover, future studies should be designed to demonstrate the direct evidence that increased methylation of the 5-HTT (SLC6A4) gene in the PFC is related to a lower binding of the radiotracer DASB to the 5-HTT.[67–71] This could be performed by using human autopsy material to gain additionally further mechanistic insights (that is, by the analysis of 5-HTT mRNA levels in different brain tissues) to confirm the accordant *in vivo* findings.[29]

CONCLUSION

The present study suggests that epigenetic changes, that is, a DNA methylation of the 5-HTTLPR, contribute even more to *in vivo* 5-HTT availability than 5-HTTLPR itself does. If confirmed, such an association between distinct (for example, CpG10) loci and prefrontal 5-HTT might explain differences in reward sensitivity, thus leading to changes in eating behavior. However, whether these changes are main drivers of weight gain and obesity and/or reversible has to be proved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The work is part of the IFB AdiposityDiseases, which is funded by the Federal Ministry of Education and Research (BMBF), Germany, FKZ: 01E01501 (http://www.bmbf.de). Results of the study were presented in part at the 2015 Annual Congress of the Society of Nuclear Medicine (SNM), Baltimore, USA, and acknowledged with the SNM Brain Imaging 3rd Place Young Investigators Award during the SNM meeting.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)