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Serotonin transporter gene hypomethylation predicts impaired antidepressant treatment response

Katharina Domschke¹, Nicola Tidow², Kathrin Schwarte², Jürgen Deckert¹, Klaus-Peter Lesch³, Volker Arolt², Peter Zwanzger² and Bernhard T. Baune⁴

¹ Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Germany

² Department of Psychiatry and Psychotherapy, University of Muenster, Germany

³ Division of Molecular Psychiatry, Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Germany

⁴ Department of Psychiatry, University of Adelaide, Australia

Abstract

Variation in the serotonin transporter gene (*5-HTT*; *SERT*; *SLC6A4*) has been suggested to pharmacogenetically drive interindividual differences in antidepressant treatment response. In the present analysis, a ‘pharmacogenetic’ approach was applied by investigating the influence of DNA methylation patterns in the *5-HTT* transcriptional control region on antidepressant treatment response. Ninety-four patients of Caucasian descent with major depressive disorder (MDD) ($n=61$) were analysed for DNA methylation status at nine CpG sites in the *5-HTT* transcriptional control region upstream of exon 1A via direct sequencing of sodium bisulfite treated DNA extracted from blood cells. Patients were also genotyped for the functional *5-HTTLPR*/rs25531 polymorphisms. Clinical response to treatment with escitalopram was assessed by intra-individual changes of HAM-D-21 scores after 6 wk of treatment. Lower average *5-HTT* methylation across all nine CpGs was found to be associated with impaired antidepressant treatment response after 6 wk ($p=0.005$). This effect was particularly conferred by one individual *5-HTT* CpG site (CpG2 (GRCh37 build, NC_000017.10 28.563.102; $p=0.002$). *5-HTTLPR*/rs25531 haplotype was neither associated with *5-HTT* DNA methylation nor treatment response. This analysis suggests that DNA hypomethylation of the *5-HTT* transcriptional control region – possibly via increased serotonin transporter expression and consecutively decreased serotonin availability – might impair antidepressant treatment response in Caucasian patients with MDD. This pharmacogenetic approach could eventually aid in establishing epigenetic biomarkers of treatment response and thereby a more personalized treatment of MDD.

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Introduction

Antidepressive pharmacotherapeutic agents such as selective serotonin reuptake inhibitors (SSRIs) have proven to be effective for a large proportion of patients in the treatment of major depressive disorder (MDD). However, about 50–60% of all patients fail to respond sufficiently to the initial treatment (Fava, 2003), which has been suggested to be partly due to genetic factors (Pare and Mack, 1971; O’Reilly et al., 1994; Franchini et al., 1998). Consequently, pharmacogenetic studies have examined association of response to antidepressants with variation in several candidate genes (cf. Kato and Serretti, 2010).

Particularly, the gene coding for the serotonin transporter (*5-HTT*; *SERT*; *SLC6A4*; chromosome 17q11.1–12), the presumed site of initial action of SSRIs, has attracted much interest. Numerous pharmacogenetic studies reported association of a functional insertion/deletion polymorphism (LPR) in the *5-HTT* transcriptional control region with antidepressant response in mood disorder patients, with, however, inconsistent findings across studies partly depending on ethnicity. Two meta-analyses of 15 and 33 studies, respectively, discerned a significant association of the less active *5-HTTLPR* S allele with impaired treatment response particularly to SSRIs in Caucasian, but not in Asian populations (Serretti et al., 2007; Porcelli et al., 2012). However, there are also contradictory studies of no influence of *5-HTT* gene variation on response to SSRIs in Caucasian patients with MDD (e.g. Lewis et al., 2011), and in none of the three available genome-wide association studies (GWAS) on antidepressant treatment response was *5-HTT* gene variation found to be associated with treatment response

Address for correspondence: Professor K. Domschke, Department of Psychiatry, University of Wuerzburg, Fuchsleinstrasse 15, D-97080 Wuerzburg, Germany.

Tel.: +49-931-20177100 Fax: +49-931-20177109.

Email: Domschke_K@ukw.de

(see meta-analysis by GENDEP Investigators, 2013). Besides ethnicity, etiological heterogeneity, high complexity of the clinically defined phenotype, environmental factors, low statistical power of the individual studies and random error in the absence of a true effect, another potential confounder of the presently available pharmacogenetic studies might be epigenetic processes such as methylation of the cytosine pyrimidine ring in CpG dinucleotides, which are flexible, temporally dynamic and functionally highly relevant mechanisms suggested to shape disease risk as well as treatment response in MDD (Menke et al., 2012; Schroeder et al., 2012; Frieling and Tadic, 2013).

The 5-*HTT* transcriptional control region contains a CpG island, which has been found to be variably methylated and to functionally influence 5-*HTT* mRNA levels with increased methylation, resulting in decreased 5-*HTT* expression depending on the 5-*HTTLPR* (Philibert et al., 2007). Four studies available so far have identified increased 5-*HTT* promoter methylation to be associated with lifetime diagnosis of depression (Philibert et al., 2008), with family history and severity of depression (Kang et al., 2013), with post-stroke depression at 2 wk and particularly at 1 yr after stroke (Kim et al., 2013), as well as with more severe depressive symptoms in a sample of monozygotic twins (Zhao et al., 2013a). In contrast, increased depressed mood in the second trimester of pregnant women was found to be associated with decreased 5-*HTT* promoter methylation in mothers as well as in their infants shortly after birth (Devlin et al., 2010).

To date, only one study explicitly investigated the impact of 5-*HTT* promoter methylation on antidepressant treatment response. In a sample of 108 Korean patients with MDD, Kang et al. (2013) failed to discern a significant influence of 5-*HTT* promoter methylation on response after 12 wk of naturalistic antidepressant treatment after correction for multiple testing, reporting, however, a trend toward higher methylation at CpG2 conferring less improvement on the Hamilton Depression scale (HAM-D). So far, only two other studies have investigated the impact of methylation patterns in other candidate genes on antidepressant treatment response (brain derived neurotrophic factor, *BDNF* (Tadic et al., 2014); interleukin-11, *IL11* (Powell et al., 2013)).

Against this background, and given that the only available study on 5-*HTT* methylation and antidepressant treatment response was performed in an Asian population (Kang et al., 2013), the present pharmacogenetic analysis was performed to further elucidate potential effects of 5-*HTT* promoter methylation on treatment response in a sample of Caucasian patients with MDD treated with escitalopram for 6 wk. We hypothesized that SSRI treatment would be less successful in patients with decreased 5-*HTT* promoter methylation, presumably counteracting SSRI action by increasing 5-*HTT* activity and thereby lowering available serotonin levels.

Method

Sample

The overall sample comprised 94 German patients treated for major depressive disorder ($f=61$; age: 47.4 ± 1.7 yr). Patients were selected for this analysis retrospectively from a larger patient cohort consecutively and naturalistically treated for MDD at the Department of Psychiatry, University of Muenster, Germany, between 2004 and 2006 (cf. Baune et al., 2008a,b; Baffa et al., 2010), when they met the criteria of treatment with escitalopram over a period of 6 wk initiated after admission for inpatient treatment. Co-medication with other psychopharmacological agents was permitted and recorded. Patients' diagnoses were ascertained by experienced psychiatrists on the basis of medical records and structured clinical interviews (SCID-I) according to the criteria of DSM-IV (Wittchen, 1997), with 12 patients meeting the criteria for melancholic depression and eight patients for atypical depression. Two participants had a comorbid anxiety disorder, which is considerably less than the expected rate of comorbidity based on epidemiological studies (Kessler et al., 1998, 1999). Patients with bipolar disorder (as retrospectively ascertained, which does not rule out conversion of pseudo-unipolar depression into bipolar disorder after the present study period), psychotic disorders including schizo-affective disorder, comorbid substance abuse disorders (apart from nicotine abuse), intellectual disability, neurological or neurodegenerative disorders impairing psychiatric evaluation and pregnant patients were not included in this analysis. Clinical characteristics were furthermore evaluated by the Hamilton Depression (HAM-D-21) scale, the Beck Depression Inventory (BDI) and the Global Assessment of Functioning (GAF) scale (see Table 1). The clinical course of depression was assessed using the HAM-D-21 scale on a weekly basis. The clinical assessment and ascertainment of HAM-D, BDI and GAF scores at admission and during the course of treatment, respectively, were done by experienced psychiatrists. Side effects were not systematically assessed in detail. Since smoking has been shown to potentially influence methylation status (Lee and Pausova, 2013; Zhao et al., 2013a,b), smoking status was ascertained in the present sample. In order to minimize the risk of ethnic stratification, Caucasian descent was ascertained by Caucasian background of both parents. The analysis was approved by the ethics committee of the University of Muenster, Germany. Written informed consent was obtained from all participating subjects, and the analysis was conducted according to the ethical principles of the Helsinki Declaration.

Medication

All patients were treated with a selective serotonin reuptake inhibitor (SSRI) (escitalopram) in a naturalistic

Table 1. Characteristics of $N=94$ patients with MDD

Characteristics	Sample ($N=94$)
Age (yr; mean \pm s.e.)	47.4 \pm 1.7
Female (N , %)	61, 64.9%
5-HTTLPR/rs25531 haplotypes	L _A /L _A : $N=29$; rest ¹ : $N=65$
Smoking status (yes vs. no; N , %)	21 vs. 73, 22.3% vs. 77.7%
HAM-D scores (mean \pm s.e.)	
Admission	21.2 \pm 0.9
Week 1	15.6 \pm 0.8
Week 6	10.4 \pm 0.6
% change at week 6 relative to week 1	-35.0% \pm 4.6
Discharge	5.4 \pm 0.5
BDI (mean \pm s.e.)	
Admission	26.5 \pm 1.0
Discharge	10.8 \pm 0.9
GAF (mean \pm s.e.)	
Admission	42.9 \pm 1.8
Discharge	68.6 \pm 2.2
Lifetime episodes of MDD (No., mean \pm s.e.)	3.1 \pm 0.4
Lifetime hospitalization (No., mean \pm s.e.)	3.2 \pm 0.3
Lifetime duration of MDD (yr, mean \pm s.e.)	12.4 \pm 1.2
Co-medication (yes; N , %)	
Antidepressant plus antipsychotics (SGA)	$N=40$; 42.6%
Antidepressant plus mood stabilizer	$N=29$, 30.9%

MDD=major depressive disorder; N =sample size; No.=number; s.e.=standard error; HAM-D=Hamilton Depression Scale (HAM-D-21); BDI=Beck Depression Inventory; GAF=Global Assessment of Functioning; yr=years; SGA=second generation antipsychotics.

¹ The category 'rest' contains haplotypes L_CL_C=2, L_AS_A=36, L_AS_G=14 and S_AS_A=13.

setting with the following combinations of antidepressant medication: escitalopram only $N=61$ (64.9%), or escitalopram plus mirtazapine $N=33$ (35.1%). In addition to antidepressants, co-medication such as atypical antipsychotics ($N=40$; 42.6% of the sample: quetiapine ($N=18$), olanzapine ($N=14$), risperidone ($N=8$)) as well as mood stabilizers ($N=29$; 30.9% of the sample: lithium ($N=20$), lamotrigine ($N=6$) (cf. Barbosa et al., 2003), valproic acid ($N=3$) (cf. Debattista et al., 2005)) was used as partly off-label augmentation of the antidepressant regimen. Co-medication was started either at the same time SSRI treatment commenced or later during the 6 wk period of observation in this study. Benzodiazepines were not used. The blood sample was taken before patients were started on the current medication. None of the included patients had received electroconvulsive therapy within 6 months before the present investigation. None of the patients received a specific psychotherapeutic intervention apart from supportive medical visits.

Response to antidepressant treatment

As the main outcome variable, clinical response to treatment was measured by the intra-individual relative (%) change of HAM-D-21 scores after week 6 relative

to HAM-D at week 1. This is in accordance with the ACNP task force guidelines on response and remission in MDD, which recommend monitoring of changes within a subject (for whom initial severity is fixed) rather than comparing response rates between subjects for whom initial values range widely (Rush et al., 2006). The initial changes in HAM-D scores occurring during week 1 were not included, as HAM-D changes during this period were most likely not related to the presently evaluated antidepressant medication (escitalopram), since in this cohort of inpatients antidepressants have been regularly switched to medication with escitalopram at the end of week 1 after initial medical and psychiatric evaluation following admission. Thus, in the present naturalistic study design HAM-D score at week 1 is to be considered the pre-therapy HAM-D-21 baseline score (cf. Baune et al., 2008a; Domschke et al., 2008, 2010). However, the statistical analysis of the proposed influence of 5-HTT DNA methylation on HAM-D %-change scores after 6 wk of treatment relative to HAM-D at week 1 as the main outcome variable (see above) using linear regression analyses was adjusted for HAM-D score at admission (see 'Statistical analysis' below). Clinical remission was defined as subjects having a HAM-D total score of 7 points or less after 6 wk of antidepressant treatment.

Isolation of DNA

DNA was isolated from human whole blood using a FlexiGene DNA Kit (QIAGEN, Germany) according to the manufacturer's instructions. In brief, lysis buffer was added to the samples and cell nuclei and mitochondria were pelletized by centrifugation. The pellet was resuspended and incubated in denaturation buffer, which contains a chaotropic salt and protease. DNA was precipitated by addition of isopropanol and washed in 70% ethanol. The dried pellet was resuspended in 25 mM TrisHCl hydration buffer, pH 7.8. DNA concentration was determined by measuring at 260 and 280 nm (GENios Pro, Tecan, Germany).

Bisulfite sequencing

Treatment of DNA with sodium bisulfite converts non-methylated cytosine to uracil, whereas methylated cytosine remains unaltered. Aliquots of isolated DNA were treated with sodium bisulfite using the EZ-96 DNA Methylation TM Kit (Zymo Research, HiSS Diagnostics GmbH, Germany) according to the manufacturer's protocol for all samples in one batch. Briefly, 450 ng of DNA were submitted to bisulfite treatment for 16 cycles at 95 °C for 30 s and 50 °C for 60 min in a thermal cycler (Mastercycler ep, Eppendorf, Wesseling-Berzdorf, Germany). DNA was eluted after desulphonation and purified using Zymo-Spin IC columns (Zymo Research, HiSS Diagnostics GmbH, Germany). As a control, commercially available fully methylated and fully non-methylated DNA was used in all experiments.

One amplicon in the *5-HTT* transcriptional control region just upstream of exon 1A (Lesch et al., 1994) was chosen for further analysis in analogy to previous studies on *5-HTT* DNA methylation with regard to stress-related measures and depression (Devlin et al., 2010; Alasaari et al., 2012; Kim et al., 2013), as well as to the only other study available so far pharmaco-epigenetically investigating *5-HTT* methylation status and antidepressant treatment response (Kang et al., 2013) (see Fig. 1). The amplicon was PCR-amplified using the following set of oligonucleotide primers recognizing bisulfite modified DNA (F: 5' TAAGGGTTTTAAGTTGAGTTA-TATTTTA 3' and R: 5' CTAATCCCRAACTAAACAAC-RAACTAA 3' (R coding for A or G), length: 635 bp, (Philibert et al., 2007)) under the following PCR conditions using Hot Start Taq polymerase (Zymo Research, HiSS Diagnostics GmbH, Germany): denaturation at 94.5 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 2 min (40 cycles). Amplified PCR products were purified with Multi Screen HTS Filter Plates (Millipore GmbH, Germany). DNA sequencing was performed using Big Dye Terminator chemistry (v.3.1, Applied Biosystems by Life Technologies, Germany) on a 3730xl DNA analyser sequencing platform (Applied Biosystems by Life Technologies, Germany). The resulting electropherograms allowed the robust identification

of methylation status of nine individual CpG sites (CpGs 1–9, further CpGs not readable due to technical sequencing failure at 5' and 3' ends of the amplicon; see Fig. 1) using Chromas (Technelysium Pty Ltd) and Sequence Scanner software (Applied Biosystems by Life Technologies, Germany). To account for run variability, all samples were tested in duplicate, yielding a mean individual methylation score for each CpG. These 9 CpGs – comprising the 5 CpGs analysed in the study by Alasaari et al. (2012), 9 CpGs analysed by Devlin et al. (2010) and all 7 CpGs analysed by Kang et al., and Kim et al., respectively (Kang et al., 2013; Kim et al., 2013) (see legend to Fig. 1) – were included in further analyses.

The obtained sequences were quantitatively analysed by determining relative peak heights (C/C+T) from the normalized sequence trace files using Epigenetic Sequencing Methylation analysis software (ESME) specifically designed and evaluated for artificially generated SNPs (C/T and G/A). This performs quality control, normalizes signals, corrects for incomplete bisulfite conversion and aligns generated bisulfite sequence and reference sequence to compare C to T peak heights at CpG sites (Lewin et al., 2004). It has been successfully used to analyse DNA methylation profiles in psychiatric phenotypes (e.g. Muschler et al., 2010; Alasaari et al., 2012; Domschke et al., 2012, 2013; Heberlein et al., 2013; Tadic et al., 2013).

Genotyping of 5-HTTLPR/rs25531

5-HTTLPR (S/L) (Lesch et al., 1996) and rs25531 (A/G) crucially modifying *5-HTTLPR* functionality (Hu et al., 2006; Wendland et al., 2006) variants were genotyped according to published protocols with minor modifications (Baune et al., 2008b; Baffa et al., 2010). DNA was amplified by PCR (60 s at 94 °C, 60 s at 64 °C, 120 s at 72 °C for 35 cycles) using oligonucleotide primers F: 5'-GGCGTTGCCGCTCTGAATGC and R: 5'-GAGGG-ACTGAGCTGGACAACCAC. PCR products were digested with HpaII at 37 °C overnight, separated for 3.5 h on 15% polyacrylamide gels and visualized by silver staining. To minimize the risk of genotyping errors, randomized probands were additionally genotyped by direct automated sequencing, which resulted in concordance rates of 100%. Genotypes were determined blind for phenotypes and independently by two investigators. Hardy–Weinberg criteria were fulfilled for genotype distributions of both investigated polymorphisms in the overall sample (*5-HTTLPR*: SS=13, SL=43, LL=38, $p(\text{Exact})=1.00$; *5-HTT* rs25531: AA=78; AG=14; GG=2, $p(\text{Exact})=0.19$). For further analyses and in analogy to preceding studies (e.g. Konneker et al., 2010; Klauke et al., 2011; Costafreda et al., 2013; Odgerel et al., 2013), *5-HTTLPR*/rs25531 haplotypes were grouped according to functionality (high-activity $L_A/L_A=29$ vs. low-activity 'rest': $L_C/L_C=2$, $L_A S_A=36$, $L_A S_C=14$, $S_A S_A=13$), since the rs25531 G allele reportedly results in reduced

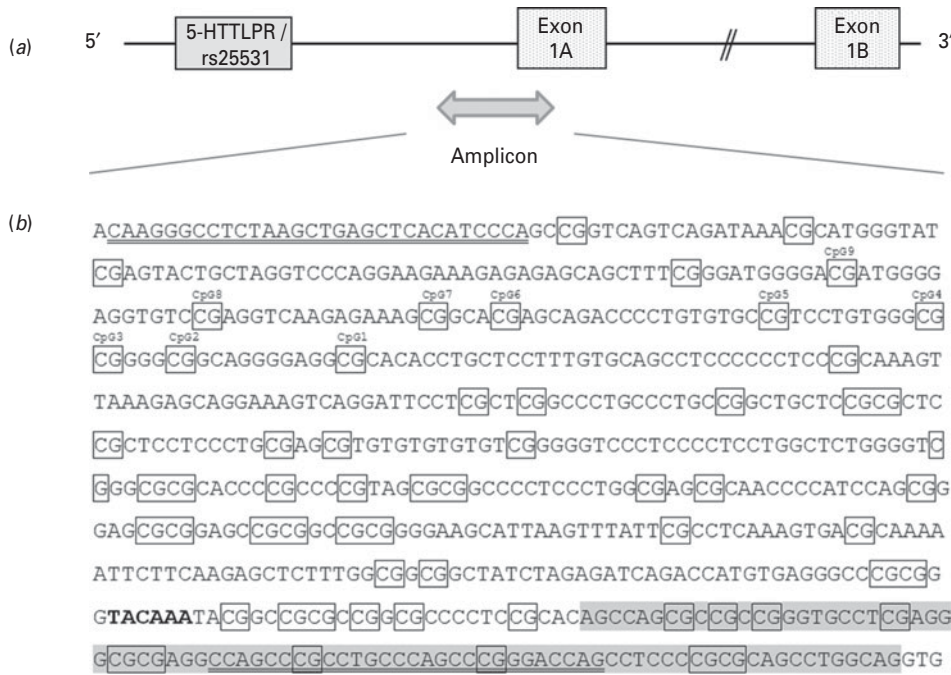


Fig. 1. Localization of analysed CpG sites in the *5-HTT* transcriptional control region. (a) The schematic *5-HTT* transcriptional control region on chromosome 17q11 is shown with exon 1A (Lesch et al., 1994), alternative exon 1B approximately 11 kb downstream (Mortensen et al., 1999), *5-HTTLPR/rs25531* and the presently analysed amplicon (not to scale; cf. Philibert et al., 2007). (b) The sequence is displayed as per GRCh37 build, National Center for Biotechnology Information (NCBI) reference sequence NC_000017.10 (28.563.286–28.562.652) and encompasses the presently analysed amplicon (primer pair binding sites double underlined) with exon 1A shadowed in grey, the presumptive TATA box in **bold** (Philibert et al., 2007) and CpG sites 1–9 analysed in the present study boxed and numbered: CpG1=Alasaari CpG1 (Alasaari et al., 2012)=Devlin CpG1 (Devlin et al., 2010)=Kang/Kim CpG 1 (Kang et al., 2013; Kim et al., 2013)=28563090 (NC_000017.10), CpG2=Alasaari CpG2=Devlin CpG2=Kang/Kim CpG 2=28563102, CpG3=Alasaari CpG3=Devlin CpG3=Kang/Kim CpG 3=28563107, CpG4=Alasaari CpG4=Devlin CpG4=Kang/Kim CpG 4=28563109, CpG5=Alasaari CpG5=Devlin CpG5=Kang/Kim CpG 5=28563120, CpG6=Devlin CpG6=28563139, CpG7=Devlin CpG7=Kang/Kim CpG 6 [sic]=28563144, CpG8=Devlin CpG8=Kang/Kim CpG 7 [sic]=CpG872 (Philibert et al., 2007)=28563160, CpG9=Devlin CpG9=28563175.

transcription levels similar to those of the *5-HTTLPR* S allele rendering only the A variant of the L allele (designated L_A) as yielding high *5-HTT* mRNA levels (Hu et al., 2006; cf. Wendland et al., 2006). Genotype and haplotype frequencies in the present sample were comparable to published frequencies in European/Caucasian populations (e.g. Wendland et al., 2006; Goldman et al., 2010; Odgerel et al., 2013).

Statistical analysis

The pharmaco-epigenetic investigations of the proposed influence of *5-HTT* DNA methylation on HAM-D %-change scores after 6 wk of treatment relative to HAM-D at week 1 as the main outcome variable (see above) were performed using linear regression analyses adjusted for age, gender, smoking status, lifetime duration of depression, lifetime hospitalizations, lifetime number of MDD episodes, HAM-D score at admission and co-medication with antipsychotics and mood stabilizers, which have previously been related to treatment response or DNA methylation alterations, respectively. Methylation

analyses were conducted using methylation data as continuous variables. In addition and for graphical purposes only, a median split was employed to illustrate response to antidepressant treatment depending on high vs. low methylation status (see Fig. 2). All covariates were included simultaneously in one model (no stepwise approach). Initially, average methylation across all 9 CpGs was used as an independent variable. Given a significant effect of average methylation on overall treatment response (percentage change of HAM-D, see above) surviving correction for multiple testing, individual CpGs within the amplicon were investigated for their effects on treatment response after 6 wk. *Post-hoc* Bonferroni correction for multiple comparisons regarding the main outcome variable (see above) set the significance level to $p \leq 0.005$ (one amplicon and nine CpGs were tested; $p = 0.05:10$). The impact of *5-HTT* DNA methylation on remission status after 6 wk was evaluated in a subsequent exploratory analysis. The present sample of 94 patients, with an assumed α error of 0.05, two-tailed test, and a low effect size of 0.15 for a single predictor in a linear multiple regression model (fixed model), achieved a *post-hoc* power of 96%.

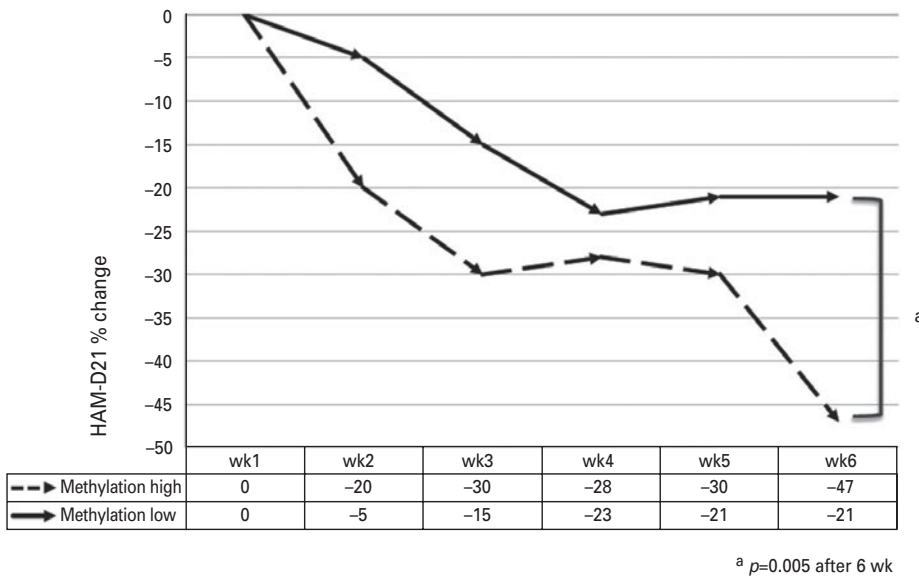


Fig. 2. Intraindividual %-change of HAM-D-21 scores during antidepressant treatment in $N=94$ patients with MDD dependent on *5-HTT* methylation. DNA methylation status 'high' (dotted line) includes values above and 'low' (continuous line) includes values below median split of average *5-HTT* methylation across all nine CpGs in the investigated transcriptional control region (low methylation group: $N=49$, mean=0.026, s.e.=0.008; high methylation group: $N=45$, mean=0.046, s.e.=0.001). Main outcome variable: Intraindividual %-change of scores on the Hamilton Depression Scale (HAM-D-21) after 6 wk of treatment relative to week 1.

Results

Sample characteristics

The mean (\pm s.e.) HAM-D score at admission was 21.2 ± 0.9 , and at discharge it was 5.4 ± 0.5 . Clinical improvement was also observed on the BDI and GAF scores between admission and discharge. The overall percentage change of the HAM-D score after 6 wk of treatment relative to HAM-D at week 1 was 35.0% (see Table 1). About 52.1% of the patients achieved remission after 6 wk of antidepressant treatment. *5-HTTLPR/rs25531* haplotype groups (L_A/L_A vs. 'rest') neither influenced clinical characteristics nor had an effect on antidepressant treatment response (data not shown).

DNA methylation status of *5-HTT*

In the whole sample, only minor average methylation across all nine CpGs was discerned in the investigated amplicon (mean \pm s.d.: 0.036 ± 0.013 , individual min: 0.012, individual max: 0.083), with levels of methylation at single CpGs varying considerably with a range (individual min-max) between 0.0 and 0.185 (CpG1: mean \pm s.d.: 0.071 ± 0.035 , min: 0.0, max: 0.165; CpG2: mean \pm s.d.: 0.059 ± 0.027 , min: 0.0, max: 0.135; CpG3: mean \pm s.d.: 0.006 ± 0.010 , min: 0.0, max: 0.045; CpG4: mean \pm s.d.: 0.045 ± 0.022 , min: 0.0, max: 0.130; CpG5: mean \pm s.d.: 0.004 ± 0.011 , min: 0.0, max: 0.075; CpG6: mean \pm s.d.: 0.005 ± 0.019 , min: 0.0, max: 0.145; CpG7: mean \pm s.d.: 0.036 ± 0.036 , min: 0.0, max: 0.185; CpG8: mean \pm s.d.: 0.043 ± 0.019 , min: 0.01, max: 0.105; CpG9: mean \pm s.d.: 0.052 ± 0.038 , min: 0.0, max: 0.180). Correlations between

individual methylation levels in all nine CpG residues calculated by pairwise correlations including Bonferroni correction ranged between 0.05 and 0.53 reaching statistical significance ($p < 0.05$) for correlation between CpG1 and CpGs 2, 3, 4 and 5 as well as between CpGs 2 and 4.

Influence of gender, age, smoking status, medication and *5-HTTLPR/rs25531* on *5-HTT* methylation

Overall *5-HTT* methylation in the analysed amplicon showed no association with gender ($p=0.71$), smoking status ($p=0.56$) or intake of medication (SSRI vs. SSRI plus mirtazapine: $p=0.96$; co-medication antipsychotics: $p=0.68$; co-medication mood stabilizers: $p=0.49$). In addition, average *5-HTT* DNA methylation ($p=0.19$) or methylation at any individual CpG site ($p=0.2-0.9$) were not associated with *5-HTTLPR/rs25531* haplotype (L_A/L_A vs. 'rest'). Only age was significantly associated with average methylation status ($p=0.005$) indicating that an increase in age was related to an increase in DNA methylation.

Influence of *5-HTT* methylation on treatment response

Influence of *5-HTT* methylation status on treatment response after 6 wk was initially investigated by running linear regression analyses with HAM-D-21%-change after 6 wk (see above) as dependent variable, average methylation across all nine CpGs in the analysed amplicon as independent variable and various covariates (age, gender, smoking status, lifetime duration of depression, lifetime hospitalizations, lifetime number of MDD episodes, HAM-D score at admission, and

co-medication with antipsychotics and mood stabilizers). Average methylation showed a significant association with antidepressant treatment response after 6 wk, with lower methylation status being associated with impaired treatment response and higher methylation with better treatment response, respectively (β , s.e. 1542.7 ± 528.5 ; $p=0.005$; surviving Bonferroni correction for multiple testing) (see Fig. 2). An exploratory analysis of 5-HTT average methylation on remission status after 6 wk showed an only nominally significant result in the same direction (β , s.e. 11.1 ± 5.3 ; $p=0.042$).

Subsequently, we extended the analyses into possible effects of methylation status at individual CpGs sites on treatment response. Among the 9 CpGs readable in this amplicon, CpG1 (GRCh37 build, NC_000017.10 28.563.090; β , s.e. 458.3 ± 225.8 ; $p=0.048$), CpG2 (GRCh37 build, NC_000017.10 28.563.102; β , s.e. 797.1 ± 244.3 ; $p=0.002$; surviving Bonferroni correction for multiple testing) and CpG4 (GRCh37 build, NC_000017.10 28.563.109; β , s.e. 611.2 ± 271.7 ; $p=0.029$) exerted (nominally) significant effects on treatment response as measured by HAM-D-21%-change after 6 wk. The results suggest that lower DNA methylation might drive worse treatment response as indicated by a lower relative reduction in HAM-D-21 score.

Discussion

The present data provide support for average DNA hypomethylation in the 5-HTT transcriptional control region – particularly conferred by CpG2 (GRCh37 build, NC_000017.10 28.563.102) – to drive impaired response to SSRI treatment in Caucasian patients with MDD. Given that in general DNA methylation of a gene's promoter region has been shown to mainly exert a silencing effect on transcriptional activity (Brenet et al., 2011) and, more specifically, increased methylation of the region encompassing the CpGs analysed in this study has been reported to be associated with decreased 5-HTT mRNA levels when controlling for 5-HTTLPR influence (Philibert et al., 2007), the presently observed detrimental effect of 5-HTT promoter hypomethylation on antidepressant treatment response could be hypothesized to be due to subsequently increased 5-HTT transcription, i.e. 5-HTT activity, and thereby decreased availability of serotonin in the synaptic cleft, which might counteract the serotonergic effects of escitalopram. In general, it is duly noted that DNA methylation patterns in peripheral biomaterial most certainly do not allow for direct conclusions regarding the respective DNA methylation patterns in brain tissue. However, rodent, rhesus monkey and human studies provide some support for a certain comparability between DNA methylation in peripheral blood cells/saliva and several brain regions (Ursini et al., 2011; Davies et al., 2012; Provencal et al., 2012; for detailed discussion see Domschke et al., 2012). Particularly with regard to

5-HTT, a recent study has demonstrated that 5-HTT promoter methylation in peripheral white blood cells was associated with lower *in vivo* measures of brain serotonin synthesis in the lateral orbitofrontal cortex as analysed by positron emission tomography (^{11}C]AMT), which suggests peripheral 5-HTT methylation as a biomarker of central serotonin function (Wang et al., 2012). However, given the primarily hypothesis-generating nature of the present pilot data, the functional consequence of 5-HTT methylation status remains highly speculative and warrant thorough investigation on mRNA and protein level in future studies.

This, particularly, as most probably an interactive effect of 5-HTT genotype and 5-HTT DNA methylation, has to be taken into account. As mentioned above, decreased 5-HTT mRNA levels have been observed, along with increased DNA methylation at 81 CpG sites in the 5-HTT promoter region against the background of 5-HTTLPR genotype (Philibert et al., 2007). In the present study, no influence of 5-HTTLPR/rs25531 haplotype on DNA methylation at the nine investigated CpG sites was discerned. However, exploratory *post-hoc* analyses suggested lower average 5-HTT DNA methylation (β , s.e. 1559.4 ± 652.5 ; $p=0.024$) and methylation of CpG2 (β , s.e. 822.0 ± 370.6 ; $p=0.035$), respectively, to confer poorer treatment response, particularly in carriers of the less active 5-HTTLPR/rs25531 haplotypes ($L_G L_G$, $L_A S_A$, $L_A S_G$ and $S_A S_A$). This finding is in line with accumulating evidence for the less active 5-HTTLPR S allele as a presumably early developmental, constitutional risk factor for impaired antidepressant treatment response in Caucasian populations (Serretti et al., 2007; Porcelli et al., 2012), and additionally points to 5-HTT DNA methylation as a potentially acquired, dynamic and more phasic determinant of serotonin transporter activity shaping overall serotonergic tone.

The result of decreased 5-HTT methylation being associated with impaired antidepressant treatment response in the present Caucasian sample is somewhat in contrast to the only other pharmaco-epigenetic study in this respect by Kang et al. (2013), who observed a trend toward increased 5-HTT methylation at CpG2 – the same CpG most significantly associated with treatment response in the present study – to confer less improvement regarding HAM-D scores in a Korean sample of patients with MDD. This divergence could be due to several reasons: (1) Asian and Caucasian populations differ greatly in 5-HTT allele frequencies (Kunugi et al., 1997; Ng et al., 2006), and ethnically different genetic association effects have been described with flip-flop phenomena with regard to the risk allele (Lin et al., 2007; Serretti et al., 2007; Porcelli et al., 2012; Myung et al., 2013), which might also apply to 5-HTT methylation status; (2) pharmacotherapy of the sample investigated by Kang et al., was considerably more heterogeneous (amitriptyline, bupropion, escitalopram, fluoxetine, imipramine, mirtazapine, paroxetine, sertraline, venlafaxine) than that in

the present analysis (escitalopram with or without mirtazapine); and (3) duration of treatment differed between the two analyses (Kang et al.: 12 wk, present study: 6 wk).

A limiting aspect of the present analysis is the sample size ($N=94$), which – while comparable with sample sizes of the three pharmaco-epigenetic studies in depression available so far ($N=39$ (Tadic et al., 2013), $N=108$ (Kang et al., 2013), $N=113$ (Powell et al., 2013)), and obviously sufficiently large to detect methylation effects surviving conservative correction for multiple testing – still entails the need for replication in larger independent samples. Also, despite the homogeneous primary medication with escitalopram in the present sample, adjunct therapy in some cases with mirtazapine, atypical antipsychotics and mood stabilizers – particularly valproic acid as a histone deacetylase (HDAC) inhibitor inducing DNA demethylation (Detich et al., 2003) – as well as the lack of a standardized dosage regime and standardized control for plasma drug levels, could have confounded the present results. Since the present naturalistic study design – like most pharmacogenetic studies – ethically did not allow for a placebo arm, medication effects and non-medication effects on the course of major depressive disorder could not be dissected. Finally, in the present sample, comorbidity with anxiety disorders was considerably lower than the expected *ca* 25–50% based on epidemiological studies (Kessler et al., 1998, 1999), which precludes generalizability of the present findings to major depressive disorder in the general population.

In conclusion, the present analysis applying a pharmaco-epigenetic approach to investigate the role of *5-HTT* DNA methylation in mediating antidepressant treatment response for the first time in a Caucasian sample showed hypomethylation of the *5-HTT* transcriptional control region to impair response to SSRI treatment in patients with MDD, possibly via increased *5-HTT* expression and consecutively decreased serotonin availability. Given robust replication in larger samples and determination of the functional relevance of the present finding, this approach could contribute to the development of a more individualized treatment concept of MDD based on epigenetic information.

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Conflict of Interest

All authors state that they have no biomedical financial interests or potential conflicts of interest related to this manuscript to declare. All affiliations mentioned below

have no relevance to the work covered in the manuscript: KD has received speaker fees from Pfizer, Lilly and Bristol-Myers Squibb. She was a consultant for Johnson & Johnson and has received funding by Astra Zeneca. BTB is a member of advisory boards and/or gave presentations for the following companies: AstraZeneca, Lundbeck, Pfizer, Servier and Wyeth. He receives funding from the National Health and Medical Research Council (NHMRC), Australia. In the past three years, JD has received speaker's honoraria from Janssen, Bristol-Myers Squibb, Wyeth, Lundbeck, Astra Zeneca and Pfizer and grant support from Medice. PZ has received speaker fees from Pfizer, Servier, Lilly, Astra Zeneca, and Bristol-Myers Squibb, he is on the advisory board of Pfizer, is a consultant for Ironwood Pharmaceuticals and has received funding from AstraZeneca. VA is member of advisory boards and/or gave presentations for the following companies: Astra-Zeneca, Janssen-Organon, Lilly, Lundbeck, Servier, Pfizer, and Wyeth. He chaired the committee for the 'Wyeth Research Award Depression and Anxiety'. All other authors have no conflicts of interest to declare, financial or otherwise.

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