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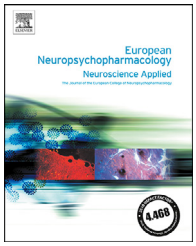

SHORT COMMUNICATION

Hypermethylation of the serotonin transporter gene promoter in panic disorder-Epigenetic imprint of comorbid depression?

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Abstract
Panic disorder (PD) is frequently comorbid with major depressive disorder (MDD), which has been associated with impaired treatment response and recovery rates. Alterations in the serotonergic system may play a crucial role in the pathogenesis of PD and MDD and might constitute a shared biological trunk of both disorders. Epigenetic patterns such as hypermethylation of the serotonin transporter gene (*SLC6A4*) have been associated with various mental disorders including MDD, but, to date, no association with PD has been reported. In the present study,

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SLC6A4 promoter methylation was investigated in two independent samples of PD patients in a case-control design (sample 1: $N = 120$; sample 2: $N = 118$), while – given the reported high comorbidity of both disorders – taking into account the effect of comorbid MDD. The functional relevance of altered *SLC6A4* promoter methylation was investigated by means of luciferase-based reporter gene assays. *SLC6A4* promoter hypermethylation in PD patients relative to healthy controls was driven by comorbid diagnosis of MDD ($p = 9 \times 10^{-6}$), whereas no altered methylation levels were observed in patients without comorbid MDD. This held true not only in comparison to healthy controls, but also in direct comparison between PD patients with and without comorbid MDD ($p = .009$). Functional analyses revealed increased methylation of the investigated region to confer decreased reporter gene activity. The present results suggest functionally relevant *SLC6A4* promoter hypermethylation as a possibly specific epigenetic marker of MDD, but not of PD itself, and thus might constitute a selective biomarker informing differential diagnosis based on individual epigenetic profiles.

1. Introduction

Panic disorder (PD) is a frequent anxiety disorder characterized by recurrent unexpected and sudden attacks of intense fear and anticipatory anxiety with a 12-month prevalence of 1.8% (Goodwin et al., 2005). PD often occurs comorbidly with mood disorders; for instance, lifetime prevalence rates for comorbid major depression (MDD) are estimated at about 30%–40% (Kessler et al., 2006).

A number of biological factors have been suggested to confer vulnerability to PD. Among those, the serotonin (5-HT) transmitter system may play a crucial role in the pathogenesis of PD as well as of a variety of other psychiatric disorders, including other anxiety disorders and MDD (Canli and Lesch, 2007). The 5-HT transporter (5-HTT), encoded by the *SLC6A4* gene, is located on presynaptic membranes and regulates intensity, duration, and distribution of serotonergic signalling by mediating the reuptake of released 5-HT from the synaptic cleft, ensuring its recycling into new vesicles. It is the prime target for selective serotonin reuptake inhibitors (SSRIs), which constitute the first-line pharmacological treatment of both anxiety disorders and MDD. On a genetic level, a length polymorphism (*5-HTTLPR*) in the upstream regulatory region of the *SLC6A4* gene has been studied extensively with regard to mental disorders (see Kenna et al., 2012; Serretti et al., 2006) but results have been equivocal, and to date no distinct risk allele for the development of anxiety disorders has been identified (see Blaya et al., 2007; Schumacher and Deckert, 2010). Recently, epigenetic mechanisms such as the methylation of cytosines in the DNA sequence have come into focus in psychiatric research since they have been shown to crucially modify gene function, to be temporally dynamic and to be sensitive to environmental influences (see Schiele and Domschke, 2018). With regard to *SLC6A4*, methylation of a CpG island surrounding the untranslated *SLC6A4* exon 1a was shown to influence *SLC6A4* mRNA levels, with hypermethylation resulting in reduced mRNA levels (Philibert et al., 2007), demonstrating the regulatory potential of this region. Furthermore, functional in vitro assays indicated that methylation of a *SLC6A4* promoter segment fused to a luciferase reporter significantly reduced reporter activity (Wang et al., 2012).

Altered – mostly increased – DNA methylation patterns of the *SLC6A4* promoter have been observed to go along with various mental disorders, particularly MDD and related phenotypes (for review see Palma-Gudiel and Fananas, 2017). In addition, there is also some evidence for an association with a family history of depression and with depressive symptomatology in monozygotic twin pairs discordant for MDD (Zhao et al., 2013). However, only few studies have investigated *SLC6A4* methylation in the context of anxiety disorders (cf. Chagnon et al., 2015), and to date no study reports on the role of *SLC6A4* methylation in PD in particular.

In the present study, we investigated *SLC6A4* methylation levels in patients with PD and healthy controls in two independent samples while – given the reported high comorbidity of anxiety disorders and depression – taking into account the effect of comorbid MDD.

2. Experimental procedures

2.1. Samples

Sample 1 consisted of 60 Caucasian patients with PD ($f = 47$; age [mean ± SD]: 34.28 ± 9.49 years) with ($N = 28$, 46.6%) or without agoraphobia, and 60 healthy controls matched by age (mean ± SD: 34.30 ± 9.25 years; $t = 0.010$, df = 118, $p = .992$) and sex ($f = 47$, $X^2 = 0.0$, df=1, $p = 1.0$). PD diagnosis was ascertained on the basis of a structured clinical interview according to DSM-IV criteria (SCID-I). The presence of current comorbid axis I diagnoses other than bipolar disorder, psychotic disorders, current alcohol dependence, current abuse of or dependence on benzodiazepines and other psychoactive substances was tolerated if PD was the primary diagnosis (depression: $N = 28$; social anxiety disorder: $N = 3$; specific phobias: $N = 2$). Further inclusion/exclusion criteria and detailed sample characteristics are described in the supplement. All patients and controls were recruited at the Department of Psychiatry, University of Würzburg, Germany, within the Collaborative Research Centre SFB-TRR-58 'Fear, Anxiety, Anxiety Disorders', project C02, and gave written informed consent prior to participation. This study was approved by the ethics committee of the University of Würzburg, Germany, and was conducted according to the ethical principles of the Helsinki Declaration.

Sample 2 comprised 59 Caucasian patients with PD ($f = 39$; age [mean ± SD] 35.83 ± 11.11 years) with ($N = 41$, 69.5%) or without agoraphobia, and 59 Caucasian healthy age- (mean ± SD: 36.54 ± 10.84 years; $t_{116} = 0.503$, $p = .616$) and sex-matched

($f = 39$, $X^2_1 = 0.0$, $p = 1.0$) controls. PD diagnosis was ascertained by experienced psychiatrists based on medical records and a structured clinical interview according to DSM-IV criteria (SCID-I). All patients were free of any current comorbid psychiatric condition except for agoraphobia (see supplement for details). All participants were recruited at the University of Münster, Germany and gave written informed consent before inclusion in the study. The study design was approved by the ethical committee of the University of Münster, Germany and conducted according to the ethical principles of the Helsinki Declaration.

2.2. *SLC6A4* methylation analysis and genotyping

Venous EDTA-blood samples were collected from all patients and controls. DNA was isolated from frozen whole blood using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany). A 635 bp amplicon comprising part of the *SLC6A4* promoter upstream of exon 1a (chr17:30,235,634-30,236,268; GRCh38.p2 Primary Assembly, UCSC Genome Browser) was chosen for DNA methylation analysis in analogy to previous studies on *SLC6A4* methylation with regard to depression and antidepressant treatment response (e.g. Devlin et al., 2010; Domschke et al., 2014; Kim et al., 2013). DNA methylation analysis is described in detail in the supplement. All samples were genotyped according to published protocols (see Schiele et al., 2016) for *5-HTTLPR* and the functionally related single nucleotide polymorphism rs25531 (see supplement for genotyping information).

2.3. Functional analysis

Functional analysis was accomplished using the pCpGfree-promoter-Lucia vector (InvivoGen, Toulouse, France) expressing a *Lucia* luciferase under a human elongation factor-1 (hEF1) promoter as described previously (Schartner et al., 2017; Schiele et al., 2018). Details regarding vectors and procedure are described in the supplement.

2.4. Statistical analysis

Differences in dimensional sample characteristics were tested by means of independent samples t-tests, differences in categorical variables by means of Chi-square tests. Differences in *SLC6A4* methylation between PD patients and controls were analysed by means of univariate ANCOVA. For confounder control, no associations emerged in either sample between average *SLC6A4* methylation and age (sample 1: $\beta = 0.070$; $p = .446$; sample 2: $\beta < 0.001$; $p = .998$), smoking status (sample 1: $t_{118} = 1.231$, $p = .221$; sample 2: $t_{53} = 0.232$; $p = .817$), intake of psychotropic medication (sample 1: $t_{118} = -0.919$, $p = .360$; sample 2: $t_{54} = -1.026$; $p = .309$; see supplementary material for details), grouped *5-HTTLPR*/rs25531 genotype (sample 1: $t_{116} = -0.877$, $p = .382$; sample 2: $t_{116} = 0.905$; $p = .327$; see supplementary material for details), or comorbid agoraphobia (sample 1: $t_{118} = -1.010$, $p = .315$; sample 2: $t_{57} = 0.275$; $p = .785$). All subsequently reported analyses are corrected for sex given a (trendwise) significant relationship between sex and average *SLC6A4* methylation in both samples (sample 1: $t_{118} = -3.125$, $p = .002$; sample 2: $t_{116} = -1.971$, $p = .051$). For functional analysis, luciferase assay data were normalized to transfection efficiency by *Renilla* luciferase control and z-transformed in order to eliminate day and measurement specific fluctuations. Differences in luciferase activity were analyzed by means of t tests. The significance level was set at $p < .05$. Bonferroni correction for multiple testing corrected the level of significance to $p < .005$ (9 CpG sites tested).

3. Results

3.1. *SLC6A4* DNA methylation in panic disorder

3.1.1. Sample 1

In the case-control comparison, significantly increased methylation at CpG sites 3, 6 and 8 (all $p \leq .005$), as well as nominally significantly higher methylation at single CpGs 2 and 4 as well as average methylation (all $p = .047$-$.002$) was observed (Table 1). However, follow-up analyses revealed that this observed hypermethylation was driven by the presence of comorbid MDD diagnosis. Methylation was significantly higher in PD patients with comorbid MDD (PD + MDD; $N = 28$) with regard to average methylation ($p = 9 \times 10^{-6}$) as well as methylation at CpG sites 2-4, 6, and 8 (all $p < .001$; Table 2, Fig. 1) as compared to healthy controls. However, PD patients without comorbid diagnoses except for agoraphobia ('pure PD'; $N = 32$) did not differ from healthy controls regarding *SLC6A4* methylation (Table 2). Relative to 'pure PD', PD + MDD patients displayed a trend for increased overall *SLC6A4* methylation ($p = .009$), particularly at CpG 2 ($p = .003$) and - on a nominally significant level - CpG sites 4, 6 and 8 (all $p < .096$; Table 2, Fig. 1).

3.1.2. Sample 2

In an independent sample comprising only 'pure PD' patients without depressive comorbidity, no significant differences were observed between patients and healthy controls with regard to average *SLC6A4* methylation as well as to methylation at single CpG sites (all $p > .173$; Table 1).

3.2. Functional analysis

Applying *in vitro* luciferase assays, non-methylated pCpGfree-promoter *Lucia_SLC6A4* vectors showed a significant increase in normalized *Lucia* luciferase activity as compared to pCpGfree-promoter *Lucia_SLC6A4* vectors methylated with M.SssI prior to transfection ($t_{16} = 8.256$, $p = 3.67 \times 10^{-7}$, Fig. 2). The pCpGfree-promoter *Lucia* vectors without insert showed no significant difference between the methylated and the non-methylated state ($t_{16} = 0.437$, $p = .668$, Fig. 2).

4. Discussion

The present study for the first time explored *SLC6A4* DNA methylation in two independent samples with panic disorder (PD) compared to healthy controls as well as the influence of comorbid diagnosis of major depressive disorder (MDD) on *SLC6A4* methylation levels. Association between increased methylation of the *SLC6A4* promoter region in PD patients relative to healthy controls was driven by comorbid diagnosis of MDD, whereas no altered methylation levels were observed in patients without comorbid MDD (i.e. 'pure PD') as confirmed in two independent samples. Importantly, this held true not only in comparison to the control group, but also in direct comparison between PD patients with and without comorbid MDD. These results corroborate previous findings failing to discern an unequivocal relationship between *SLC6A4* and anxiety disorders, particularly PD, on a

Table 1 *SLC6A4* DNA methylation levels in patients with panic disorder (PD) and matched healthy controls.

CpG	Sample 1			Sample 2		
	PD patients (mean ± SE) $N = 60$	Controls (mean ± SE) $N = 60$	Statistics[a]	PD patients (mean ± SE) $N = 59$	Controls (mean ± SE) $N = 59$	Statistics[a]
Average	0.0571 ± 0.0030	0.0420 ± 0.0033	$F = 7.20$; $p = .008$	0.0289 ± 0.0021	0.0265 ± 0.0024	$F = 1.18$; $p = .280$
1	0.0899 ± 0.0053	0.0715 ± 0.0064	$F = 1.98$; $p = .162$	0.0414 ± 0.0063	0.0336 ± 0.0060	$F = 1.07$; $p = .303$
2	0.0511 ± 0.0045	0.0407 ± 0.0039	$F = 4.01$; $p = .047$	0.0211 ± 0.0033	0.0209 ± 0.0036	$F = 0.00$; $p = .992$
3	0.0624 ± 0.0036	0.0409 ± 0.0038	$F = 8.96$; $p = .003$	0.0430 ± 0.0058	0.0342 ± 0.0049	$F = 1.36$; $p = .246$
4	0.0396 ± 0.0034	0.0284 ± 0.0027	$F = 4.61$; $p = .034$	0.0218 ± 0.0036	0.0189 ± 0.0033	$F = 0.12$; $p = .727$
5	0.0169 ± 0.0025	0.0096 ± 0.0019	$F = 3.75$; $p = .055$	0.0177 ± 0.0038	0.0113 ± 0.0028	$F = 1.25$; $p = .266$
6	0.0281 ± 0.0031	0.0153 ± 0.0025	$F = 10.38$; $p = .002$	0.0097 ± 0.0023	0.0072 ± 0.0020	$F = 1.88$; $p = .173$
7	0.0697 ± 0.0065	0.0528 ± 0.0061	$F = 1.07$; $p = .303$	0.0229 ± 0.0039	0.0225 ± 0.0040	$F = 0.23$; $p = .636$
8	0.0908 ± 0.0059	0.0618 ± 0.0060	$F = 8.08$; $p = .005$	0.0433 ± 0.0055	0.0465 ± 0.0059	$F = 0.03$; $p = .865$
9	0.0655 ± 0.0052	0.0573 ± 0.0054	$F = 0.37$; $p = .545$	0.0396 ± 0.0049	0.0431 ± 0.0060	$F = 0.00$; $p = .989$

[a] *p*-values from univariate ANOVA are reported with average DNA methylation or methylation at the respective single CpG sites as dependent variable and group (healthy controls vs. PD patients) and sex as fixed factors. SE: standard error of the mean.

Table 2 *SLC6A4* DNA methylation levels in patients with panic disorder (PD) with and without comorbid major depression (MDD) and matched healthy controls (sample 1).

CpG	Controls (mean ± SE) $N = 60$	'pure PD' (mean ± SE) $N = 28$	PD+MDD (mean ± SE) $N = 32$	Statistics[a]	*t*-test Controls vs. 'pure PD'[b]	*t*-test Controls vs. PD + MDD	*t*-test 'pure PD' vs. PD + MDD
Average	0.0420 ± 0.0033	0.0500 ± 0.0044	0.0652 ± 0.0035	$F = 5.98$; $p = .003$	$t = -1.44$; $p = .152$	$t = -4.80$ $p = 9 \times 10^{-6}$	$t = 2.71$; $p = .009$
1	0.0715 ± 0.0064	0.0823 ± 0.0078	0.0986 ± 0.0070	$F = 2.32$; $p = .102$	-	-	-
2	0.0407 ± 0.0039	0.0389 ± 0.0058	0.0650 ± 0.0061	$F = 6.76$; $p = .002$	$t = 0.26$; $p = .796$	$t = -3.45$; $p = .001$	$t = 3.11$; $p = .003$
3	0.0409 ± 0.0038	0.0583 ± 0.0052	0.0671 ± 0.0047	$F = 5.16$; $p = .007$	$t = -2.69$; $p = .009$	$t = -4.01$; $p = 1.05 \times 10^{-4}$	$t = 1.25$; $p = .217$
4	0.0284 ± 0.0027	0.0334 ± 0.0056	0.0466 ± 0.0050	$F = 3.16$; $p = .046$	$t = -0.81$; $p = .422$	$t = -3.46$; $p = .001$	$t = 1.74$; $p = .088$
5	0.0096 ± 0.0019	0.0116 ± 0.0031	0.0230 ± 0.0038	$F = 2.69$; $p = .073$	-	-	-
6	0.0153 ± 0.0025	0.0233 ± 0.0044	0.0336 ± 0.0041	$F = 7.24$; $p = .001$	$t = -1.59$; $p = .119$	$t = -3.93$; $p = 1.71 \times 10^{-4}$	$t = 1.69$; $p = .096$
7	0.0528 ± 0.0061	0.0556 ± 0.0095	0.0857 ± 0.0077	$F = 3.01$; $p = .053$	-	-	-
8	0.0618 ± 0.0060	0.0811 ± 0.0090	0.1020 ± 0.0072	$F = 4.77$; $p = .010$	$t = -1.83$; $p = .070$	$t = -4.30$; $p = 6 \times 10^{-5}$	$t = 1.82$; $p = .074$
9	0.0573 ± 0.0054	0.0656 ± 0.0076	0.0654 ± 0.0072	$F = 0.20$; $p = .823$	-	-	-

[a] *p*-values from univariate ANOVA are reported with average DNA methylation or methylation at the respective single CpG sites as dependent variable and group (healthy controls vs. 'pure PD' patients vs. PD + MDD patients) and sex as fixed factors.
[b] *t*- and *p*-values from post-hoc tests are reported; SE: standard error of the mean.

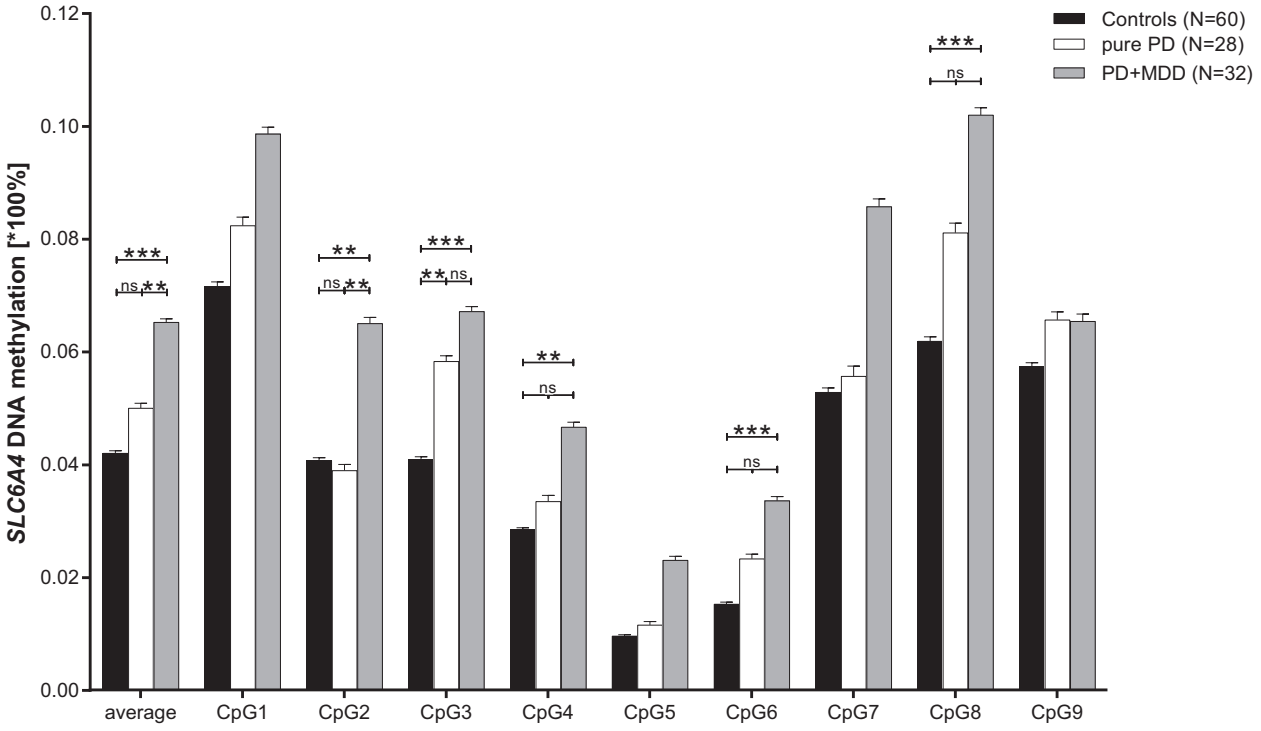

Fig. 1 *SLC6A4* DNA methylation in PD patients with or without comorbid MDD (Sample 1).
PD: panic disorder; MDD: major depressive disorder; * $p < .05$; ** $p < .01$; *** $p < .001$.

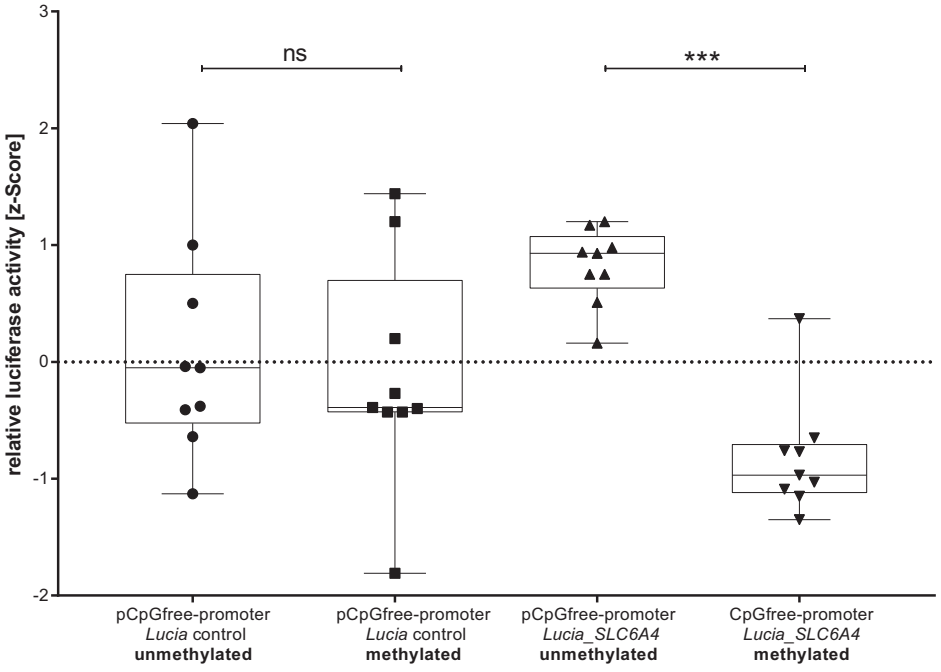

Fig. 2 Functional analysis of *SLC6A4* promoter DNA methylation using luciferase-based reporter gene assays.
No significant difference in normalized reporter gene activity was discerned between methylated or non-methylated pCpGfree-promoter *Lucia* control vectors lacking the insert of the sequence spanning CpGs 1-9 (left). Normalized reporter gene activity was significantly decreased in the presence of pCpGfree-promoter *Lucia_SLC6A4* vectors containing the methylated insert spanning CpGs 1-9 compared to those carrying a non-methylated insert (right); *** $p < .001$, ns: not significant.

genetic and epigenetic level (Blaya et al., 2007; Chagnon et al., 2015; Schumacher and Deckert, 2010), and are additionally in line with evidence for *SLC6A4* hypermethylation to be associated with MDD and related phenotypes (for review see Palma-Gudiel and Fananas, 2017). Furthermore, in a functional in vitro assay, increased methylation of the investigated gene regulatory region was shown to confer decreased reporter gene activity, which corroborates a previous finding on *SLC6A4* methylation affecting transcriptional activity (Wang et al., 2012) and is in accordance with evidence for *SLC6A4* hypermethylation to be associated with decreased brain 5-HT synthesis in the lateral left and right orbitofrontal cortex (Wang et al., 2012) as well as with lowered 5-HTT availability in prefrontal cortex regions (Drabe et al., 2017). Thus, *SLC6A4* promoter hypermethylation may constitute a distinctive epigenetic signature of comorbid MDD in PD, but not of PD itself, potentially conferred by decreased transcriptional activity of the *SLC6A4* promoter region.

The present results add to the recently burgeoning evidence for a role of epigenetic alterations in the pathogenesis of mental disorders and may aid in the search for valid biomarkers for precision phenotyping guiding the differential diagnostic process and potentially also informing clinical decision making and therapeutic options. Given that group sizes (with/without comorbid MDD) were relatively small, replication in larger samples is warranted, probing the potential of *SLC6A4* promoter hypermethylation as a selective differential diagnostic marker of MDD and additionally exploring possible implications for therapeutic action considering comorbidity profiles. In the latter regard, differential *SLC6A4* promoter methylation has previously been demonstrated to predict response to SSRI treatment in depression (Domschke et al., 2014) and to be related to successful CBT response in pediatric anxiety disorders (Roberts et al., 2014). Given that comorbid anxiety and depression is associated with poorer outcomes and a higher proportion of treatment resistance (Bystritsky, 2006; Fava, 2003), it seems pertinent to address fine tuning of treatment approaches for mental health multimorbidity informed by epigenetic information. Also, since MDD is highly comorbid not only with PD but also other anxiety and mental disorders, the potential of *SLC6A4* methylation status to serve as a diagnostic tool to separate MDD diagnosis from other diagnoses should be addressed in future studies including comparison to non-comorbid MDD cases. These studies might also want to further explore a potentially sexually dimorphic role of *SLC6A4* methylation given the presently observed significant relationship between sex and average *SLC6A4* methylation as described previously (Palma-Gudiel et al., 2019) as well as the impact of life events applying an (epi)gene-environment approach (cf. Palma-Gudiel and Fananas, 2017) and disentangle the differential effects of *SLC6A4* methylation on anxiety vs. depression vs. other mental disorders by investigating disorder-specific intermediate phenotypes. Moreover, since localized changes in methylation patterns might reflect global changes in genomic DNA methylation either by CpG hyper- and hypomethylation, epigenome-wide association studies (EWAS) are warranted taking into account comorbidity profiles in larger, sufficiently powered samples of PD patients. A general limitation of the present study pertains to the fact that DNA methylation was measured in samples derived from peripheral blood, which may encompass cell type composition effects driven by a vast amount of factors including inflammation, diet, exercise, stress or hormonal status as possible confounders. Moreover, no data on *SLC6A4* gene expression level were available for both cohorts. Thus, despite being located within a CpG island previously found to influence *SLC6A4* mRNA levels (see introduction, Philibert et al., 2007) and given previous results from functional in vitro *assays* indicating that *SLC6A4* promoter methylation significantly reduced reporter activity (Wang et al., 2012) as replicated in the present study, the exact functional relevance of the presently investigated CpGs on SLC6A4 mRNA- and protein level remains to be elucidated. Along these lines, peripheral methylation or gene expression patterns do not necessarily allow for deductions regarding methylation and expression status in brain tissue. However, *SLC6A4* methylation in peripheral cells was shown to be associated with hippocampal volume in depression (Booij et al., 2015) as well as to be inversely related to brain 5-HT synthesis (Drabe et al., 2017; Wang et al., 2012) providing some evidence for peripheral *SLC6A4* methylation as a proxy of central 5-HT function. Also, while smoking status was statistically controlled for, information regarding smoking status was not available for the control group in sample 2, which could have confounded the respective results.

In sum, the present results suggest functionally relevant *SLC6A4* promoter hypermethylation as a possibly specific epigenetic marker of MDD, but not of PD itself, and thus might constitute a selective biomarker facilitating differential diagnosis and potentially even inform treatment strategies based on individual epigenetic profiles.

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Conflicts of interest

VA has received compensations for his contributions as member of advisory boards and for presentations for the following companies: Astra-Zeneca, Eli Lilly, Janssen-Cilag, Lundbeck, Otsuka, Servier, and Trommsdorff. These cooperations have no relevance to the work that is covered in the manuscript. All other authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Miriam A. Schiele: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Leonie Kollert:** Investigation, Writing - review & editing. **Klaus-Peter Lesch:** Conceptualization, Funding acquisition, Writing - review & editing. **Volker Arolt:** Supervision, Writing - review & editing. **Peter Zwanzger:** Supervision, Writing - review & editing.

Jürgen Deckert: Conceptualization, Funding acquisition, Resources, Writing - review & editing. **Christiane Ziegler:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Katharina Domschke:** Conceptualization, Funding acquisition, Project administration, Resources, Software, Validation, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.07.131.

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