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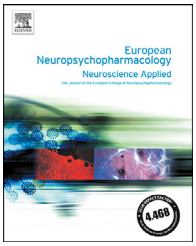
European Neuropsychopharmacology (2020) 36, 154–159



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SHORT COMMUNICATION

DNA methylation in the 5-HTT regulatory region is associated with CO_2-induced fear in panic disorder patients

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Received 15 November 2019; received in revised form 22 April 2020; accepted 28 April 2020

KEYWORDS
Panic attacks;
Serotonin transporter;
Epigenetics;
Carbon dioxide

Abstract
A polymorphism in the gene encoding the serotonin (5-HT) transporter (5-HTT) has been shown to moderate the response to CO_2 inhalation, an experimental model for panic attacks (PAs). Recurrent, unpredictable PAs represent, together with anticipatory anxiety of recurring attacks, the core feature of panic disorder (PD) and significantly interfere with patients' daily life. In addition to genetic components, accumulating evidence suggests that epigenetic mechanisms, which regulate gene expression by modifying chromatin structure, also play a fundamental role in the etiology of mental disorders. However, in PD, epigenetic mechanisms have barely been examined to date. In the present study, we investigated the relationship between methylation at the regulatory region of the gene encoding the 5-HTT and the reactivity to a 35% CO_2 inhalation in PD patients. We focused on four specific CpG sites and found a significant association be-

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https://doi.org/10.1016/j.euroneuro.2020.04.011

tween the methylation level of one of these CpG sites and the fear response. This suggests that the emotional response to CO_2 inhalation might be moderated by an epigenetic mechanism, and underlines the implication of the 5-HT system in PAs. Future studies are needed to further investigate epigenetic alterations in PD and their functional consequences. These insights can increase our understanding of the underlying pathophysiology and support the development of new treatment strategies.

1. Introduction

Panic attacks (PAs) are characterized by intense fear and physical symptoms, and strongly impair patients' quality of life (Davidoff et al., 2012). Unexpected PAs represent the core symptom of panic disorder (PD) and are now listed as a severity specifier applicable to all mental disorders in the Diagnostic and Statistical Manual of Mental Disorders (DSM)−5 (American Psychiatric Association, 2013).

PAs can be reliably provoked in PD patients using a brief 35% CO_2-inhalation, and interestingly also in healthy individuals when the CO_2 dosage is increased (for review see Leibold et al., 2015). This suggests the existence of a CO_2-sensitivity continuum in which PD patients are hypersensitive to CO_2. By now, CO_2-hypersensitivity is a well-validated endophenotype of PD. A series of studies linked this endophenotype to the serotonergic (5-HT) system. Briefly, tryptophan depletion to reduce 5-HT levels increased the panic response to CO_2-inhalation (Schruers et al., 2000). In contrast, acute administration of the 5-HT precursor to increase 5-HT levels reduced the response to CO_2 (Schruers et al., 2002). Next, natural variation in 5-HT levels was studied by focusing on functional polymorphisms that alter gene expression. Participants homozygous for the long (L)-allele of the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) reported the highest fear to CO_2 (Schruers et al., 2011). The L-allele has been linked to a higher 5-HTT expression compared to the short (*S*)-allele (Lesch et al., 1996). In the brain, the 5-HTT is located on the presynaptic neuron and a higher expression leads to a relatively more efficient reuptake of 5-HT into the neuron, thereby affecting neurotransmission.

Research in the last few years has shown that not only static genetic variants contribute to how an individual's brain network is activated and works, but that gene expression is also influenced by environmental cues, mediated through dynamic epigenetic modifications (Guo et al., 2011). Genetically identical individuals can have a distinct risk to develop a disorder, as they have different gene expression and thus differently functioning networks caused by these epigenetic modifications. One of the most studied types of epigenetic regulation is DNA methylation occurring at cytosine residues. Although DNA methylation has gained attention in recent years, few studies have examined epigenetic modifications in PD. Only two genome-wide association studies (Iurato et al., 2017; Shimada-Sugimoto et al., 2017) and, based on evidence that the 5-HT system dysfunction is implicated in mental disorders, some candidate gene-based studies, focusing on crucial factors of the 5-HT system such as the monoamine oxidase A (*MAO-A*) gene, attempted to determine the involvement of DNA methylation in the etiology of PD (Domschke et al., 2012; Ziegler et al., 2016). Interestingly, although variation in the gene encoding the 5-HTT (*SLC6A4*) has been associated with PD and CO_2-sensitivity, its methylation status has not yet been specifically examined in this context.

The aim of this study was to address this gap and to explore the relationship between DNA methylation in the regulatory region of the *SLC6A4* gene and CO_2-responsivity in PD patients, taking 5-HTT genotype into account. Previously, we showed that 5-HTTLPR L-allele carriers, presumably having a relatively higher expression of 5-HTT, displayed a stronger response to CO_2 (Schruers et al., 2011). Based on this, we hypothesized that relatively lower DNA methylation levels, generally considered to be concomitant with a higher gene transcription and thus 5-HTT expression, were associated with higher CO_2-reactivity.

2. Experimental procedures

All experimental procedures were approved by the Medical Ethics Committee of Maastricht University and Maastricht University Hospital, the Netherlands, and were conducted in accordance with the Declaration of Helsinki. Participants gave written informed consent.

2.1. Participants

Caucasian PD patients were recruited from the Academic Anxiety centre Maastricht ($n = 153$, mean age 37.58 ± 10.94 years, 63 males). An experienced clinician verified PD (with(out) agoraphobia) as primary diagnosis using DSM-IV criteria and the Mini International Neuropsychiatric Interview (Sheehan et al., 1998). Additionally, a brief medical examination took place. Exclusion criteria were a history of or current pulmonary or cardiovascular disease or cerebral aneurysm, severe hypertension (>170 mmHg/100 mmHg), epilepsy, current pregnancy, and psychotropic medication except for antidepressants. Mean PD duration was 11.23 ± 10.69 years; 110 patients were smokers.

2.2. CO_2 inhalation and assessment

Patients took a single vital capacity breath of 35% CO_2 (SOL Nederland BV, Landgraaf, the Netherlands) and held their breath for 4 s according to a standardized protocol in our laboratory (Schruers et al., 2000). The response was assessed before and after the inhalation using the Visual Analogue Scale for Fear (VAS-F, ranging from not at all to very severe) and the 13-item Panic Symptom List (PSL, ranging from absent to very severe).

TGGGGAGGTGTCCGAGGTCAAGAGAAAGCGGCACGAGCAGACCCCTGTGTGCCGTCCTGTGGGCG[1]CG[2]GGGCG[3]

GCAGGGGAGGCG[4]CACACCTGCTCCTTTGTGCAGCCTCCCCCCTCCCGCAAAGTTAAAGAGCAGGAAAGTCAGGAT

Fig. 1 Sequence overview of the investigated CpG sites (bold/gray boxes) in the *SLC6A4* gene. Primer sequences (underlined) for PCR amplification covered chr17:30,236,007-30,236,154 (GRCh38/hg38). The sequencing primer is double underlined.

2.3. Genetic and epigenetic analyses

Saliva was collected 20 min before the CO_2-inhalation (Oragene self-collecting tubes, DNA Genotek Inc, Ontario, Canada). Genomic DNA was extracted using the AutoGenFlex DNA isolation system according to manufacturer's instructions (Autgen, Hilliston, MA, USA). 5-HTTLPR genotype (LL, LS, SS) was determined as previously described (Schruers et al., 2011). In addition to the length polymorphism, a single nucleotide polymorphism within 5-HTTLPR (rs25531) leading to an L-allele (L_G) with similar 5-HTT expression levels as the S-allele (S_A) was determined. This genotype was clustered according to expression levels (LL, LS, SS).

For epigenetic analyses, DNA was treated with sodium bisulfite according to manufacturer's instructions (Epitect96 Bisulfit Kit, Qiagen, Hilden, Germany). The target region was amplified using polymerase chain reaction (PCR, HotStar Taq Plus Polymerase, Qiagen) followed by sequencing-by-synthesis pyrosequencing of the target sequence (PyroMark Q96 MD^{TM} Pyrosequencer, Qiagen). The percentage of non-converted cytosines relative to an expected 100% signal was assessed per CpG site using the Pyro Q-CpG^{TM} Software (Qiagen). Samples were run in duplicate with a maximum difference of 5% between duplicates. The mean of each duplicate was used for analyses. To test for PCR-bias and incomplete bisulfite-conversion, fully methylated, 50%-methylated and non-methylated DNA samples were included. Primers were designed using PyroMark Assay Design 2.0 Software (Qiagen) with a focus on four CpG sites in the 5-HTT regulatory region (GRCH38/hg38 CpG1=chr17:30,236,091; CpG2=chr17:30,236,089; CpG3=chr17:30,236,084; CpG4=chr17:30,236,072; Fig. 1). These sites were chosen based on the previously reported association between methylation and mental health (Domschke et al., 2014). The potential functional relevance of these sites was investigated using ENCODE (Encode Project Consortium, 2012) and ORegAnno in the UCSC Genome Browser (GRCh37/hg19, February 2009).

2.4. Statistical analyses

The effect of DNA methylation (treated as continuous variable) on CO_2-induced fear and panic symptoms was analyzed using linear regression, adjusted for age, sex, smoking status, and 5-HTTLPR/rs25531 genotype. These variables were included *a priori* based on potential effects on methylation and/or CO_2-reactivity. Outcome variables were change in fear and panic symptoms, calculated by post- minus pre-inhalation ratings. Analyses were done using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA). After Bonferroni correction for four comparisons, statistical significance was set to $p<0.0125$.

3. Results

Overall, methylation levels of the four investigated CpG sites were low. Individual percentages ranged from 0% to 8.74%. Mean methylation (± SD) was as follows: CpG1 2.30±1.63, CpG2 3.64±1.94, CpG3 1.46±1.46, and CpG4 3.05±1.89. Linear regression revealed a significant negative association between CpG3 methylation level and changes in fear scores in PD patients (p=0.004, B=−3.729, Beta=−0.233, t=−2.922) (Table 1). CpG1, CpG2 and CpG4 were not significantly associated with fear scores ($p>0.0125$). Regarding panic symptoms, no effect was found for any CpG site ($p>0.0125$).

Table 1 Effect of CpG1 to CpG4 methylation on CO_2-induced fear and panic symptoms. p-values: uncorrected values, Beta: standardized coefficients, *denotes statistical significance.

	Fear		Panic symptoms	
	p-value	Beta	p-value	Beta
Corrected for length polymorphism				
CpG1	0.748	0.027	0.745	0.027
CpG2	0.134	−0.122	0.588	−0.044
CpG3	**0.004***	−0.233	0.733	−0.028
CpG4	0.698	−0.032	0.835	0.017
Corrected for single nucleotide polymorphism				
CpG1	0.793	0.021	0.867	0.014
CpG2	0.127	−0.123	0.632	−0.039
CpG3	**0.004***	−0.230	0.819	−0.019
CpG4	0.622	−0.041	0.880	0.012

4. Discussion

This study showed a significant negative association between CO_2-induced fear and the degree of CpG3 methylation (chr17:30,236,084) in the regulatory region of the *SLC6A4* gene in PD patients, in line with our hypothesis. In our previous genetic study, we observed a higher fear response to CO_2 in 5-HTTLPR LL-allele carriers (Schruers et al., 2011) who presumably have a higher 5-HTT expression. The 5-HTT is located on presynaptic 5-HT neurons and is responsible for reuptake of extracellular 5-HT. After reuptake, 5-HT is no longer available to bind to postsynaptic receptors. Therefore, regulating 5-HT reuptake regulates 5-HT action. A higher amount of 5-HTT is associated with a relatively faster reuptake of 5-HT, and thus a relatively faster termination of 5-HT signaling, when compared to a low amount of 5-HTT. Recent evidence shows that gene function is not just regulated by genetic variation, but also by long-lasting epigenetic modifications such as DNA methylation (Narayan and Dragunow, 2010). Generally, DNA methylation modifies chemical characteristics of DNA and consequently chromatin conformation. In the context of gene promoters, DNA methylation is mainly associated with

gene transcription repression (Weber et al., 2007). Therefore, we suggest that increased methylation in the investigated promoter region of the *SLC6A4* gene is directly or indirectly associated with decreased 5-HTT protein expression. Accordingly, high methylation levels at the gene promoter would be associated with a relatively lower 5-HTT expression and in turn a relatively slower 5-HT reuptake. 5-HT activates various receptors, including 5-HT1A and 5-HT2A. In the dorsal periaqueductal gray, 5-HT1A receptor activation was shown to inhibit glutamatergic neurons, while 5-HT2A receptor activation stimulates GABAergic neurons. Through these pathways, 5-HT is thought to inhibit panic-/escape-like behavioral and physiological responses (Paul et al., 2014). These observations in rodents are consistent with the negative association found in the present study, with higher methylation being associated with a milder fear response (small change in fear scores).

To determine the functional relevance of the identified CpG site, we made use of the ENCODE and ORegAnno databases. No annotated transcription factor binding sites could be identified. Previous research links individual CpG site methylation in the investigated gene to, e.g., in vivo 5-HTT availability (Drabe et al., 2017). It can be speculated that the identified CpG site in our study could have similar functional effects, which however remains to be investigated.

As few studies have examined DNA methylation in PD, the investigated CpG sites in this study were based on the previously reported association between methylation and mental health (Domschke et al., 2014). In that study, lower DNA methylation across all nine investigated CpGs was associated with impaired antidepressant treatment response, with one individual CpG site seemingly mediating this effect (CpG2). The CpG site that was significantly associated with the emotional response to CO_2 in our study (CpG3) corresponds to CpG1 in Domschke's study. This discrepancy might be explained by a different focus, namely reported depression symptoms in patients with major depressive disorder and CO_2-reactivity in PD patients. Nevertheless, combined, this data suggests that the investigated region could have biological relevance in mental health. While two previous genome-wide association studies comparing PD patients and healthy individuals did not provide support for a role of the *SLC6A4* gene in PD (Iurato et al., 2017; Shimada-Sugimoto et al., 2017), the results were limited by small sample sizes and not controlling for factors such as smoking (Iurato et al., 2017). We addressed these limitations in the present study and moreover focused on CO_2-sensitivity as endophenotype, which is considered to depend on less genes than a complex disorder (Gottesman and Gould, 2003). This approach can increase the likelihood of detecting links between a genetic variant, gene expression and the susceptibility to a disorder. In this respect, epigenetic mechanisms are of particular interest, as they are at the basis of gene regulation and can be altered by environmental stimuli such as stressful life events, thereby representing the link between genes and environmental exposures. This interplay shapes the risk for mental disorders. A better understanding of how epigenetic modifications play a role in diseases could result in more personalized treatments. For example, it could be speculated that PD patients with low CpG3 methylation would benefit from increasing methylation at this site to decrease their CO_2-sensitivity. To support this notion, a longitudinal study would be useful to determine whether distinct changes in DNA methylation in fact are a cause of CO_2-hyperreactivity, as the current study was an association study and does not allow drawing conclusions on causality. Following first-degree relatives of PD patients, who are at higher risk to develop the disorder (Leibold et al., 2015), over many years and repeatedly assessing (genome-wide) methylation levels and testing their CO_2-reactivity could provide important insights into the link between methylation changes and the development of CO_2-hyperreactivity and the disorder. While a longitudinal study can relate specific events and their sequence to an outcome, measurements over a prolonged period are needed. Therefore, sample sizes have to be large to account for potential drop out over time. These factors make such a study design highly costly and challenging. An alternative approach is to manipulate the epigenetic machinery in rodents. We previously validated CO_2-exposure as cross-species model for panic (Leibold et al., 2016). Central infusion of pharmacological compounds that alter enzymes involved in adding or removing methylation marks, or the use of epigenetic editing, could shed light onto the consequences of methylation changes in the *SLC6A4* gene. Another valuable future direction into the dynamic nature of methylation would be assessing the effects of therapy on methylation levels in the *SLC6A4* gene, as done for other genes affecting 5-HT metabolism such as *MAO-A* (Ziegler et al., 2016). After successful cognitive behavioral therapy *MAO-A* gene hypomethylation increased to levels comparable to those in healthy controls, which emphasizes the reversibility of risk patterns. Additionally to examining changes in DNA methylation, investigating whether baseline methylation could serve as biomarker to predict who could benefit most from a particular treatment would also be a significant step forward in tailoring individualized treatment approaches.

The results of this study should be interpreted with keeping some limitations in mind. First, as the primary tissue of interest (i.e. brain) was inaccessible, DNA methylation levels were measured peripherally. The proportion of epithelial cells and leucocytes varies between individuals, which can affect results (Smith et al., 2015). Nevertheless, DNA methylation in saliva epithelial cells might still serve as a proxy for the brain, as exemplified by a study demonstrating hypomethylation of the catechol-O-methyltransferase gene promoter in both saliva and post-mortem brain samples from schizophrenia and bipolar disorder patients (Nohesara et al., 2011). Further, other studies showed similar DNA methylation patterns comparing saliva and blood (Staunstrup et al., 2017), and blood-brain correlations (Murphy et al., 2005). Second, while the sample size exceeded many other candidate gene studies, replication is warranted in larger samples to account for variation in saliva cell composition.

In conclusion, this is the first study showing that CO_2-sensitivity in PD patients might be mediated by altered epigenetic regulation of the *SLC6A4* gene. Determining the functional relevance can increase our understanding into the underlying molecular basis and can eventually contribute to developing more personalized treatments.

CRediT authorship contribution statement

N.K. Leibold: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **M.T. Weidner:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing. **C. Ziegler:** Methodology, Writing - review & editing. **G. Ortega:** Investigation, Methodology, Writing - review & editing. **K. Domschke:** Methodology, Writing - review & editing. **K.P. Lesch:** Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing. **D.L. Van den Hove:** Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing. **K.R. Schruers:** Conceptualization, Project administration, Supervision, Writing - review & editing.

Role of funding source

Klaus-Peter Lesch (KPL) and his team are supported by the Deutsche Forschungsgemeinschaft (DFG: CRU 125, CRC TRR 58 A1/A5, No. 44,541,416), the European Union's Horizon 2020 Research and Innovation Programme under Grant No. 728,018 (Eat2beNICE), ERA-Net NEURON/RESPOND,No. 01EW1602B, and 5–100 Russian Academic Excellence Project. Katharina Domschke (KD) received funding by the German Research Foundation (DFG) – project no. 44,541,416 – TRR 58, subprojects C02 and Z02. The funders had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

KRS designed the study and wrote the protocol. NKL did the experiments. NKL, MTW, CZ, OG, designed and did the epigenetic analysis. NKL and MTW undertook the statistical analysis, and NKL wrote the first draft of the manuscript. DvdH, KD, and KPL contributed to overall discussion. All authors contributed to and have approved the final manuscript.

Conflict of interest

KPL served as a speaker for Eli Lilly and received research support from Medice, and travel support from Shire, all outside the submitted work. Other authors declare no conflict of interest.

Acknowledgement

None.

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