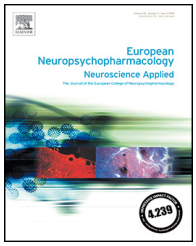
European Neuropsychopharmacology (2019) 29, 171–178

www.elsevier.com/locate/euroneuro



Smoking moderates association of 5-HTTLPR and in vivo availability of serotonin transporters

Michael N. Smolka[a,1,*], **Matthias Reimold**[b,1], **Andrea Kobiella**[a], **Gerald Reischl**[c], **Marcella Rietschel**[d], **Andreas Heinz**[e]

[a] *Department of Psychiatry and Neuroimaging Center, Technische Universität Dresden, Würzburger Straße 35, Dresden 01187, Germany*
[b] *PET Center, Department of Nuclear Medicine, University of Tübingen, Tübingen, Germany*
[c] *Department of Preclinical Imaging and Radiopharmacy, University of Tübingen, Tübingen, Germany*
[d] *Department of Genetic Epidemiology, Central Institute of Mental Health, Mannheim, Germany*
[e] *Department of Psychiatry, Charité University Medicine Berlin, Campus Charité Mitte, Berlin, Germany*

Received 15 March 2017; received in revised form 4 August 2018; accepted 7 August 2018

KEYWORDS
5-HT transporter;
DASB PET;
Smoking;
5-HTTLPR

Abstract
Although preclinical studies clearly indicate an effect of 5-HTTLPR genotype on 5-HT transporter (5-HTT) expression, studies in humans provided inconclusive results, hypothetically due to environmental factors and differences in individual behavior. For example, nicotine and other constituents of tobacco smoke elevate serotonin (5-HT) levels in the brain and may thereby cause homeostatic adaptations in 5-HTT availability that moderate effects of 5-HTTLPR genotype. To test whether 5-HTT availability in the midbrain is affected by smoking status and 5-HTTLPR genotype, we pooled data from prior studies on in vivo 5-HTT availability (BP_{ND}) measured with positron emission tomography (PET) and [^{11}C]DASB. In total, we reanalyzed 5-HTT availability in 116 subjects using ANCOVA statistics. ROI analysis revealed that current smokers and non-smokers do not differ in midbrain BP_{ND}. Interestingly, smoking status significantly interacted with 5-HTTLPR genotype: active smoking was associated with reduced 5-HTT availability only in LL subjects but not in carriers of the S-allele. From the perspective of genotype effects, non-smokers showed the expected association with 5-HTTLPR, i.e. higher 5-HTT availability in LL subjects compared to carriers of the S-allele, whereas this pattern was actually reversed for active smokers. Our study indicates that smoking status moderates the association of 5-HTTLPR

* Corresponding author.
E-mail address: michael.smolka@tu-dresden.de (M.N. Smolka).
[1] Both the authors contributed equally to this work.

https://doi.org/10.1016/j.euroneuro.2018.08.509

genotype and 5-HTT expression, which may help to explain inconsistent findings in previous studies. Regarding the mechanism, we suggest that smoking may induce epigenetic processes such as methylation of *SLC6A4,* which can differ depending on its genetic constitution.

1. Introduction

Smoking was the second leading risk factor for early death and disability worldwide in 2015 (GBD 2015 Risk Factors Collaborators, 2016). Cigarette smokers die about 10 years younger than non-smokers (Doll et al., 2004), and although mostly well aware of the detrimental consequences, smokers frequently fail to quit. Several lines of evidence indicate that nicotine's ability to affect emotionality may be critical for the understanding of mechanisms promoting nicotine use: Studies indicate that nicotine has anxiolytic, mood enhancing and stress dampening effects (reviewed in Picciotto et al., 2002), and even seems to reduce risk for recurrence of major depression (Glassman et al., 2001), suggesting one major motivation to smoke is to regulate emotional states. These effects might at least in part be mediated by serotonergic neurotransmission, which affects a wide range of behaviors from food intake and reproductive activity to sensory processing, motor activity, cognition and emotion (Canli and Lesch, 2007; Heinz et al., 2011). And indeed, several lines of evidence indicate that the emotional effects of smoking might at least in part be due to its effects on serotonin (5-HT) neurotransmission: Tobacco smoke and nicotine enhance 5-HT neurotransmission by inhibition of 5-HT reuptake (Rausch et al., 1989), stimulation of 5-HT release (Rausch et al., 1989; Ribeiro et al., 1993) and inhibition of MAO-A activity (Fowler et al., 1996). Further evidence for enhanced 5-HT neurotransmission in smokers is provided by a study that used auditory stimulus processing to assess cortical effects of serotonergic inputs (Gallinat et al., 2005).

5-HT neurotransmission is regulated by presynaptic 5-HT transporters (5-HTT), which remove 5-HT from the synapse. To compensate for increased 5-HT neurotransmission in the smoker's brain, the density of 5-HTTs might be upregulated, as indicated by some studies that investigated the effect of smoking on in vivo 5-HTT availability in humans: Using Single Photon Emission Computed Tomography (SPECT), Staley et al. (2001) reported that brainstem [^{123}I]β-CIT uptake was modestly (10%) higher in male (but not female) smokers compared to non-smokers. A comparable sex x smoking interaction was also reported for the diencephalon (Ruhe et al., 2009). In a study on the effects of acute alcohol withdrawal, the Yale group reported a significant interaction between heavy alcohol use and smoking status when assessing 5-HT transporter availability in both the diencephalon and brainstem (Cosgrove et al., 2009). Specifically, smoking compared to non-smoking was associated with greater 5-HTT availability in drinkers, whereas 5-HTT availability was not affected by smoking status in controls. Nevertheless, these pioneer studies have some limitations:

First, the SPECT radioligand [^{123}I]β-CIT lacks pharmacological specificity and binds to both DA and 5-HT transporters, thereby complicating ROI measurements in brain regions where both transporters are localized (Heinz et al., 2004). Moreover, the poor spatial resolution of SPECT limits the distinction of brainstem nuclei such as the substantia nigra from the dorsal raphe (van Dyck et al., 2004). Thus, brainstem binding of [^{123}I]β-CIT does not allow to measure 5-HTT availability with high selectivity.

Second, [^{123}I]β-CIT competes with endogenous serotonin for 5-HTT binding (Heinz et al., 2004). Thus, individual differences in synaptic serotonin release and turnover can interact with [^{123}I]β-CIT and higher in vivo availability of 5-HTT might be due to increased density of 5-HTT or decreased 5-HT levels in the synaptic cleft. Indeed, a study that investigated the association of BMI with 5-HTT expression using [^{11}C]DASB, a radioligand not displaced by endogenous serotonin (Hummerich et al., 2004) did not find any differences between smokers and non-smokers (Erritzoe et al., 2010).

Third, variations of the promoter region of the 5-HTT gene (5-HTTLPR) have not been accounted for, although 5-HTTLPR affects 5-HTT expression and function as shown by in vitro studies (Lesch et al., 1996; Hu et al., 2006). In line with this hypothesis, some SPECT and Positron Emission Tomography (PET) studies observed reduced 5-HTT availability in human carriers of the S-allele of 5-HTTLPR (Heinz et al., 2000; Reimold et al., 2007a; Praschak-Rieder et al., 2007; Kalbitzer et al., 2010). However, other PET and SPECT studies failed to report an association between 5-HTTLPR and in vitro 5-HTT availability (Jacobsen et al., 2000; Willeit et al., 2001; Shioe et al., 2003; Parsey et al., 2006; Murthy et al., 2010). These inconsistencies may reflect that beyond the genetic impact, 5-HTT levels are affected by numerous additional factors, including environmental influences like daylight (Praschak-Rieder et al., 2008; Kalbitzer et al., 2010) and stress hormone regulation (Reimold et al., 2011).

Lastly, Heinz et al. (2000) reported a drug x genotype interaction and observed increased 5-HTT brainstem availability in homozygous healthy carriers of the L-allele, while alcohol-dependent subjects carrying this genotype displayed reduced 5-HTT availability in the brainstem. Among carriers of the S-allele, 5-HTT availability did not differ between control and alcoholic subjects. A recent report of our group found a similar interaction of smoking status with 5-HTTLPR genotype (Kobiella et al., 2011): Here, smoking compared to non-smoking LL individuals showed reduced availability, whereas smoking was not associated with 5-HTT availability in carriers of the S-allele.

Therefore, we wanted to further explore the hypothesis that smoking effects on 5-HTT availability are modulated by serotonin transporter genotype. For this investigation, we pooled data from prior studies of our group on in vivo 5-HTT availability (BP_{ND}) measured with PET and [^{11}C]DASB, a highly selective 5-HTT ligand that is not affected by endogenous serotonin levels (Hummerich et al., 2004), and reanalyzed data of 116 participants.

Table 1 Sample characteristics.

	Non-smokers ($N=64$)	Smokers ($N=52$)	Total ($N=116$)	P
Sex (females)	28 (44%)	21 (40%)	49 (42%)	0.715[1]
Age (years)	43 ± 9	42 ± 8	43 ± 9	0.574[2]
Genotype 5-HTTLPR (LL)	22 (34%)	15 (29%)	37 (32%)	0.525[1]
Groups				
Controls	40 (63%)	35 (67%)	75 (65%)	0.013[1]
Alcohol dependence	5 (8%)	12 (23%)	17 (15%)	
OCD	12 (19%)	2 (4%)	14 (12%)	
Depression	7 (11%)	3 (6%)	10 (9%)	

OCD = obsessive-compulsive disorder
[1] χ^2 Test;
[2] T-Test;

2. Experimental procedures

2.1. Subjects

In this study, we reanalyzed data of subjects that underwent [^{11}C]DASB PET in former research projects of our group and that were published in five prior reports (Reimold et al., 2007a; 2007b; 2008; 2011; Kobiella et al., 2011). In total, 116 volunteers were included in this re-analysis (mean $\pm$ SD age: 43.0 ± 8.7 years; 49 of them females); 52 of these probands were current smokers and 64 were non-smokers (48 subjects who never smoked and 16 who stopped smoking at least 12 months before scanning).

Among these probands, 75 were healthy volunteers without any psychiatric axis I or II disorder (SCID I & II-interview; First et al., 1997; 2001), 17 were alcohol dependent, 14 suffered from obsessive-compulsive disorder (OCD) and 10 had a diagnosis of acute major depression according to ICD-10 and DSM-IV. OCD patients and patients with major depression or alcohol dependence had no other psychiatric axis I disorder and no history of drug dependence (except alcohol for the respective patients) or current drug abuse (random urine drug testing and SCID-interview; First et al., 2001) except for nicotine. Alcohol dependent patients were abstinent from alcohol for at least 10 days and less than 30 days. Some patients received prior treatment with various antidepressants or medication for alcohol withdrawal, but as controls, were free of any psychotropic medication for at least 7 days and five half-lives of the respective drug prior to the PET scan. Participants with neurological disorders, liver or kidney disease, or immune deficiency were excluded.

Diagnostic groups were differently distributed between non-smokers and smokers ($\chi^2 = 10.8$, df = 3; $p = 0.013$, compare Table 1). As expected, alcohol dependence was more frequent in current smokers than in non-smokers (23% vs. 8%), whereas OCD was most frequent in non-smokers compared to in current smokers (19% vs. 4%). Neither age ($t = 0.564$; df = 114; $p = 0.574$) nor sex ($\chi^2 = 0.133$; df = 1; $p = 0.715$) differed between smoker groups.

Urine and breath testing was performed to exclude current alcohol or drug consumption except for tobacco use. The studies were approved by the Ethics Committees of the University of Heidelberg and Tübingen and were in accordance with the Helsinki Declaration. Informed written consent was obtained from all participants.

2.2. Genetic constitution of the 5-HTT promoter

DNA was isolated from whole blood by standard techniques. 5-HTTLPR variants were determined with PCR techniques. PCR amplification of 5-HTTLPR was as follows: in a total volume of 20 μl, ~25 ng of genomic DNA was amplified with the primers as reported previously (Wendland et al., 2006): forward 5′-TCCTCCGCTTTGGCGCCTCTTCC-3′ and reverse 5′-TGGGGGTTGCAGGGGAGATCCTG-3′, in the presence of 1 × Promega PCR Master Mix (Promega Corporation, Madison, WI, USA), without multiplexing. PCR conditions were: 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 90 s at 70 °C, 60 s at 72 °C, and a final extension of 10 min at 72 °C. Detection of rs25531 by restriction digest was performed as follows: 20 μl PCR product was digested with 10 Units HpaII (New England BioLabs, Ipswich, MA, USA) for 4 h at 37 °C. Samples were run on 3% agarose gel and precast stained with GelRed (Biotium, Hayward, CA, USA). To score the genotypes, undigested and digested PCR products of the same sample were run on adjacent lanes as described previously (Wendland et al., 2006). Genotyping revealed that 37 subjects were homozygous for the high transcribing L allele (LL), 79 subjects were carriers of at least one low transcribing S allele (SL/SS). Genotype distribution did not differ significantly smoking groups ($\chi^2 = 1.267$, df = 2; $p = 0.531$, compare Table 1).

2.3. PET methods

[^{11}C]DASB synthesis, image reconstruction and processing and ROI analysis including kinetic modeling have been described previously (Reimold et al., 2007a; Kobiella et al., 2011). In brief, after intravenous bolus injection of 20 mCi [^{11}C]DASB, cerebral radioactivity distribution was measured over 90 min using a GE Advance PET scanner (GE Medical Systems, Milwaukee, WI, USA). Images were realigned and stereotactically normalized with Matlab (Version 7, MathWorks, Sherborn, MA, USA) and SPM2 (Wellcome Department of Imaging Neuroscience). The midbrain region of interest had been created by applying an isocontour threshold of BP_{ND} 2.5 to a group of healthy subjects to allow for a robust quantification of the most part of serotonergic transporters in the brainstem. It is primarily located in the midbrain, but also contains adjacent parts of the pons and, particularly, includes in large part the raphe. The ROI for thalamus, putamen, amygdala and cerebellum (reference tissue) had been defined manually in Montreal Neurological Institute (MNI) space upon anatomical criteria. 5-HTT availability $BP_{ND} = k_3/k_4$, a measure proportional to the concentration of binding sites (Innis et al., 2007) was calculated with the multilinear reference tissue model 2 (Ichise et al., 2003). The methodology was the same for all patients.

All smoking participants were allowed to smoke ad libitum till start of the PET measurement, and in fact all smoking participants did so. Thus, smokers were abstinent for about 30 min when the PET measurement started

Table 2 Effects of smoking status and 5-HTTLPR genotype on midbrain 5-HTT availability.

Source	df	F	p	Partial η^2
Corrected model	3	2.757	0.046	0.069
Intercept	1	1793.389	0.000	0.941
5-HTTLPR (LL vs. SS/SL)	1	0.002	0.965	0.000
Smoking status (non-smoker, smoker)	1	1.875	0.174	0.016
5-HTTLPR * smoking status	1	7.917	0.006	0.066

2.4. Statistical analysis

Statistical analyses were calculated with SPSS 23.0 for Windows (IBM Corp, Armonk, NY, USA). 5-HTT availability (absolute BP_{ND} values) in the midbrain VOI as measured with [^{11}C]DASB PET was the dependent variable of interest for all analyses. ANCOVA was conducted to test the hypothesis that both smoking and 5-HTTLPR LL genotype are associated with increased 5-HTT availability and to test possible interactions between these two factors. To control for effects of potentially confounding variables, we conducted four further ANOVAs, including age and gender as additional covariates, examining healthy controls only and finally also including duration of daylight as a potential confounder (Kalbitzer et al., 2010) in the full sample.

3. Results

ANOVA statistics with smoking status and genotype as factors explained a significant amount of inter-individual variance in midbrain 5-HTT availability ($F = 2.757$; $df = 3$; $p = 0.046$; $\eta^2 = 6.9\%$, see Table 2). This model neither revealed a significant impact of smoking status nor genotype (SS/SL < LL), however, the interaction between smoking status and genotype was substantially associated with 5-HTT availability (cf. Fig. 1): Smokers compared to non-smokers showed lower 5-HTT availability in the midbrain only when being homozygous for the L-allele (18% lower; $F = 6.358$; $df = 1$; $p = 0.013$; $\eta^2 = 5.4\%$), while carriers of the 5-HTTLPR S-allele displayed no significant association between 5-HTT availability and smoking status (7% higher in smokers; $F = 1.672$; $df = 1$; $p = 0.199$; $\eta^2 = 1.5\%$).

From the perspective of 5-HTTLPR genotype, our data indicate that non-smokers show the expected association between 5-HTT availability and genotype, i.e. higher 5-HTT availability in LL subjects than in carriers of the S-allele (14% higher; $F = 4.803$; $df = 1$; $p = 0.030$; $\eta^2 = 4.1\%$), whereas the pattern was actually reversed for active smokers (13% lower in LL subjects; $F = 3.336$; $df = 1$; $p = 0.070$; $\eta^2 = 2.9\%$).

To cross-validate the finding of differential effects of smoking status on 5-HTT availability depending on 5-HTTLPR genotype, we computed four additional ANOVA models controlling for possible confounds by age, sex, disorder group, or duration of daylight. In the first ANCOVA we included age and sex. As in the simple 2 × 2 design, smoking status and 5-HTTLPR genotype per se did not explain a significant amount of variance in 5-HTT availability, while the interaction of both factors remained significant ($p = 0.006$; $\eta^2 = 6.6\%$). In a second step, to exclude confounding due to the heterogeneity in the total sample of 116 participants, we computed an ANCOVA containing age, sex and group, as well as a 2 × 2 ANOVA restricted to healthy controls only. Again, in both analyses, smoking status and genotype per se did not explain a significant amount of variance in 5-HTT availability, while the interaction of both factors did ($p = 0.011$; $\eta^2 = 5.9\%$ for all participants, and $p = 0.008$; $\eta^2 = 9.5\%$ for healthy controls only, cf. Supplementary Material Table S1 and Fig. S1). In a final step we conducted an ANCOVA with smoking status, genotype and duration of daylight to control for possible seasonal effects on midbrain 5-HTT availability. Once again, smoking status and genotype per se did not explain a significant amount of variance in 5-HTT availability, while the interaction of both factors did ($p = 0.030$; $\eta^2 = 4.2\%$).

We also conducted exploratory analyses of ROIs in the thalamus and the striatum (compare Supplementary Material Tables S2/3 and Figs. S2/3). While we were able to replicate the statistical interaction between smoking and genotype in the thalamus at a trend level ($F = 3.250$, $df = 1$,

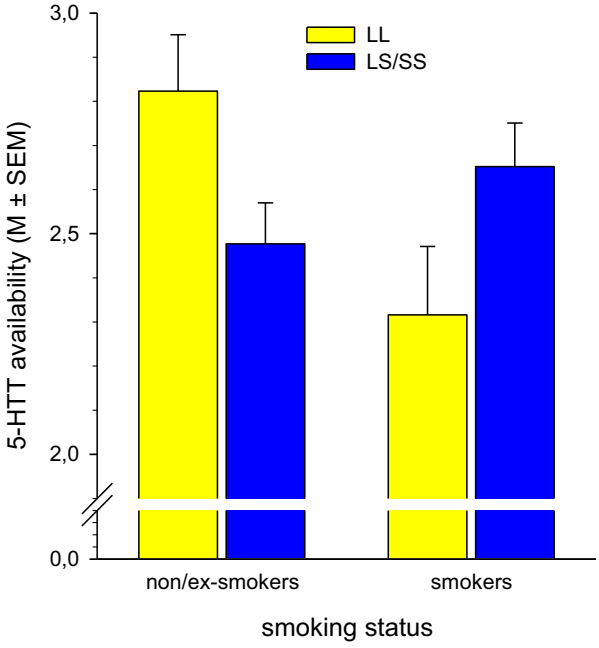


Fig. 1 Effects of smoking status and 5-HTTLPR genotype on midbrain 5-HTT availability.
5HTT availability in non-smoking LL participants ($n = 22$) was $M = 2.82$, $SD = 0.66$ and $M = 2.48$, $SD = 0.54$ compared to non-smoking S carriers ($n = 42$), i.e. 14% higher. 5HTT availability in smoking LL participants ($n = 15$) was $M = 2.32$, $SD = 0.63$ compared to $M = 2.65$, $SD = 0.62$ in smoking S carriers ($n = 37$), i.e. 13% lower.

$p = 0.074$), we did not find it in the putamen ($F = 1.089$, $df = 1$, $p = 0.299$).

4. Discussion

The main finding of our study is that non-smoking carriers of the 5-HTTLPR S allele compared to those homozygous for the L allele show the expected decrease in vivo availability of 5-HTT in the midbrain, whereas current smokers display a numerically reversed pattern but no significant genotype differences. Availability of 5-HTT in this study was measured with the radioligand [^{11}C]DASB, which binds selectively to 5-HTT and whose 5-HTT binding is not altered by changes in endogenous serotonin (Hummerich et al., 2004), suggesting that smoking status moderates the association between genotype and in vivo expression of 5-HTT in the midbrain. Thus, depending on the ratio of smokers and non-smokers in a sample, results regarding the association of 5-HTTLPR with 5-HT expression should differ, which may be one reason for inconsistent findings in previous studies.

Regarding a possible main effect of smoking, we were not able to replicate a general increase in 5-HTT availability in smokers as measured with [^{123}I]β-CIT SPECT (Staley et al., 2001). In fact, when pooling over genotypes, smokers showed numerically even lower [^{11}C]DASB binding in midbrain than non-smokers. Indeed, our null finding is in accordance with the study of Erritzoe and coworkers (2010) that used the same radioligand ([^{11}C]DASB). This may be due to differences in radioligand competition with endogenous serotonin, which was described for [^{123}I]β-CIT but not [^{11}C]DASB (Heinz et al., 2004; Hummerich et al., 2004). Thus, although smoking is thought to enhance 5-HT neurotransmission (for a review see Olausson et al., 2002; Gallinat et al., 2005) by stimulating 5-HT release (Rausch et al., 1989; Ribeiro et al., 1993), inhibiting its reuptake (Rausch et al., 1989) and decreasing MAO-A activity (Fowler et al., 1996), smoking per se does not appear to substantially affect 5-HTT expression in general. Likewise, smoking was also not associated with neural availability of 5-HT$_{2A}$ receptors as measured with [^{18}F]altanserin PET (Erritzoe et al., 2009).

Our key finding is an interaction between smoking status and 5-HTTLPR genotype, which was driven by differential effects of smoking depending on genotype: Specifically, an association of active smoking with reduced 5-HTT availability in the midbrain was limited to LL subjects, whereas smoking did not modulate 5-HTT availability in carriers of the S-allele. This finding resembles the pattern of genotype x alcohol interaction found in detoxified alcohol-dependent patients and healthy controls (Heinz et al., 2000). Our findings thus confirm early reports of lower 5-HTT expression and function in S-allele versus LL carriers (Lesch et al., 1996; Heinz et al., 2000; Reimold et al., 2007a) but limit this observation to non-smokers only. The lower expression of serotonin transporters in smoking subjects with the 5-HTLPR LL genotype was first described by Kobiella et al. (2011) in a subsample of 54 subjects also included in our new study with a much larger sample size ($n = 116$). Reasons for such genotype-specific interactions are unknown but may include 5-HTTLPR effects on smoking behavior and neuroprotective factors including BDNF (Ohmoto et al., 2013; Chou et al., 2013). A previous study found a similar interaction between 5-HTTLPR genotype and alcohol dependence (Heinz et al., 2000). The authors hypothesized that a specific susceptibility towards neurotoxic effects of excessive alcohol use in individuals homozygous for the L allele may result in reduced 5-HT neurotransmission and consecutive down-regulation of transporters. Because smoking rates in alcohol dependent subjects are 3- to 4-fold higher than in controls, the interaction observed in the former study may have been primarily due to smoking and not to drinking. In any case, the underlying neuro-pharmacological mechanisms remain unknown and deserve further elucidation.

One possible mechanism to be studied is that 5-HTTLPR genotype-associated smoking behavior (Ohmoto et al., 2013) induces epigenetic mechanisms (review in Alegria-Torres et al., 2011), which might interact with the effect of 5-HTTLPR on 5-HTT expression. Indeed, epigenetic research elucidated that *SLC6A4* mRNA transcription, i.e. expression of 5-HTT, is regulated by both genetic and epigenetic mechanisms (Philibert et al., 2008). In this context, methylation of the CpG island of *SLC6A4* was significantly associated with mRNA production, and increased amounts of methylation at these residues were associated with decreased mRNA transcription (Philibert et al., 2008). Importantly, methylation state of CpG islands of *SLC6A4* assessed in monocytes and T cells from peripheral blood was also found to be associated with lower in vivo 5-HT synthesis in the orbitofrontal cortex as measured with [^{11}C]AMT PET (Wang et al., 2012) as well as with higher depression scores (assessed with the BDI) in a large sample of monozygotic twin pairs (Zhao et al., 2013). Thus, epigenetic research indicates substantial neurochemical and behavioral effects of the methylation state of *SLC6A4*. To our knowledge, to date no study explicitly investigated the effect of smoking on methylation of *SLC6A4*, however, some reports indicate strong effects of smoking on methylation of various genes (Alegria-Torres et al., 2011). Of interest, smokers showed lower methylation of the promoter of the *MAOA* gene compared to non-smokers (Philibert et al., 2010), and methylation state was found to be associated with MAO-A activity in the brain as measured by [^{11}C]clorgyline PET (Shumay et al., 2012). Since MAO-A catabolizes 5-HT, smoking might thus alter 5-HT neurotransmission and 5-HTTLPR expression indirectly via epigenetic mechanisms. Therefore, we suggest to test whether smoking affects methylation of *SLC6A4*, and to investigate how methylation of *SLC6A4* and *MAOA* affect 5-HTT expression in vivo. Moreover, to explain the observed interaction between smoking status and 5-HTTLPR, differential effects of genotype on methylation of *SLC6A4* remain to be explored. Recent investigations indeed reported that effects of psychosocial stressors on methylation state of *SLC6A4* are moderated by 5-HTTLPR genotype (Vijayendran et al., 2012; Beach et al., 2014).

In summary, our study indicates that the association of active smoking with reduced midbrain availability of 5-HTT is limited to 5HTTLPR LL subjects, whereas smoking does not significantly modulate 5-HTT availability in carriers of the S-allele. Thereby, active smokers showed a reversed pattern regarding the effects of 5-HTTLPR, i.e. numerically lower 5-HTT availability in LL subjects compared to carriers of the S-allele, whereas non-smokers showed the expected pattern of significantly higher 5-HTT expression in

LL- versus S-carriers. To elucidate the mechanisms by which smoking moderates the association of 5-HTTLPR genotype and 5-HTT expression, further research should investigate the effects of 5-HTTLPR on smoking patterns, their respective effects on epigenetic mechanisms regulating *SLC6A4* expression, and their interaction with variation in this gene. A better understanding of the complex interplay between genetic variation and epigenetic processes as well as individuals' behavior might help to elucidate the mechanisms that underlie the pivotal role of the 5-HT system for mood, cognition and mental health (Caspi et al., 2010; Heinz et al., 2011; Lesch and Waider 2012).

Conflict of interest

The authors declare no conflict of interest.

Contributors

MNS, MRe and AH designed the study. Data acquisition was done by MRe and AK. GR was responsible for radiosyntheses. MRi was responsible for genotyping. MNS, MRe, AK analysed data. MNS, MRe, AK, MRi and AH were involved in interpretation of data.

The manuscript was drafted by MNS and AH. All authors revised the manuscript for important intellectual content and have approved the final version.

Role of funding sources

This study was supported by the Deutsche Forschungsgemeinschaft (DFG, grants HE 2597/4-3, HE 2597/7-3, RE 1472/6-2, SM 80/2-2, SM 80/5-1, SM 80/5-2, SM 80/7-1, SM 80/7-2, SFB 940/1 and SFB 940/2. The DFG had no further 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.

Acknowledgments

The authors like to thank Jens Treutlein for genotyping; Derik Herman for help with participant information, Anke Zimmer-Butz and Dr. Dorothea Elisabeth Ulshöfer for assistance during data collection, and Karl Mann for general support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2018.08.509.

References

Alegria-Torres, J.A., Baccarelli, A., Bollati, V., 2011. Epigenetics and lifestyle. Epigenomics 3 (3), 267–277.

Beach, S.R., Dogan, M.V., Brody, G.H., Philibert, R.A., 2014. Differential impact of cumulative SES risk on methylation of protein-protein interaction pathways as a function of SLC6A4 genetic variation in African American young adults. Biol. Psychol. 96, 28–34.

Canli, T., Lesch, K.P., 2007. Long story short: the serotonin transporter in emotion regulation and social cognition. Nat. Neurosci. 10 (9), 1103–1109.

Caspi, A., Hariri, A.R., Holmes, A., Uher, R., Moffitt, T.E., 2010. Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. Am. J. Psychiatry 167 (5), 509–527.

Chou, Y.H., Chen, C.K., Wang, S.J., Tsai, S.J., Lirng, J.F., Liou, Y.J., Lin, C.L., Yang, K.C., Lin, M.W., Lo, W.C., Liao, M.H., Chen, C.C., 2013. Association of brain serotonin transporter availability and brain-derived neurotrophic factor in models of serotonin transporter genotypes in healthy subjects. J. Clin. Psychopharmacol. 33 (3), 432–435.

Cosgrove, K.P., Krantzler, E., Frohlich, E.B., Stiklus, S., Pittman, B., Tamagnan, G.D., Baldwin, R.M., Bois, F., Seibyl, J.P., Krystal, J.H., O'Malley, S.S., Staley, J.K., 2009. Dopamine and serotonin transporter availability during acute alcohol withdrawal: effects of comorbid tobacco smoking. Neuropsychopharmacology 34 (10), 2218–2226.

Doll, R., Peto, R., Boreham, J., Sutherland, I., 2004. Mortality in relation to smoking: 50 years' observations on male British doctors. BMJ 328 (7455), 1519.

Erritzoe, D., Frokjaer, V.G., Haahr, M.T., Kalbitzer, J., Svarer, C., Holst, K.K., Hansen, D.L., Jernigan, T.L., Lehel, S., Knudsen, G.M., 2010. Cerebral serotonin transporter binding is inversely related to body mass index. Neuroimage 52 (1), 284–289.

Erritzoe, D., Frokjaer, V.G., Haugbol, S., Marner, L., Svarer, C., Holst, K., Baare, W.F., Rasmussen, P.M., Madsen, J., Paulson, O.B., Knudsen, G.M., 2009. Brain serotonin 2A receptor binding: relations to body mass index, tobacco and alcohol use. Neuroimage 46 (1), 23–30.

First, M.B., Spitzer, R.L., Gibbon, M., Williams, J.B.W., 1997. Structured Clinical Interview for DSM-IV Personality Disorders, (SCID-II). American Psychiatric Press, Inc., Washington, D.C.

First, M.B., Spitzer, R.L., Gibbon, M., Williams, J.B.W., 2001. Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition. (SCID-I/P). Biometrics Research, New York State Psychiatric Institute, New York.

Fowler, J.S., Volkow, N.D., Wang, G.J., Pappas, N., Logan, J., Shea, C., Alexoff, D., MacGregor, R.R., Schlyer, D.J., Zezulkova, I., Wolf, A.P., 1996. Brain monoamine oxidase: a inhibition in cigarette smokers. Proc. Natl. Acad. Sci. U. S. A. 93 (24), 14065–14069.

Gallinat, J., Kunz, D., Lang, U.E., Kalus, P., Juckel, G., Eggers, J., Mahlberg, R., Staedtgen, M., Wernicke, C., Rommelspacher, H., Smolka, M.N., 2005. Serotonergic effects of smoking are independent from the human serotonin transporter gene promoter polymorphism: evidence from auditory cortical stimulus processing. Pharmacopsychiatry 38 (4), 158–160.

GBD 2015 Risk Factors Collaborators, 2016. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the global burden of disease study 2015. Lancet 388 (10053), 1659–1724.

Glassman, A.H., Covey, L.S., Stetner, F., Rivelli, S., 2001. Smoking cessation and the course of major depression: a follow-up study. Lancet 357 (9272), 1929–1932.

Heinz, A., Jones, D.W., Mazzanti, C., Goldman, D., Ragan, P., Hommer, D., Linnoila, M., Weinberger, D.R., 2000. A relationship between serotonin transporter genotype and in vivo protein expression and alcohol neurotoxicity. Biol. Psychiatry 47 (7), 643–649.

Heinz, A., Jones, D.W., Zajicek, K., Gorey, J.G., Juckel, G., Higley, J.D., Weinberger, D.R., 2004. Depletion and restoration of endogenous monoamines affects beta-CIT binding to serotonin but not dopamine transporters in non-human primates. J. Neural Transm. Suppl. 68, 29–38.

Heinz, A.J., Beck, A., Meyer-Lindenberg, A., Sterzer, P., Heinz, A., 2011. Cognitive and neurobiological mechanisms of alcohol-related aggression. Nat. Rev. Neurosci. 12 (7), 400–413.

Hu, X.Z., Lipsky, R.H., Zhu, G., Akhtar, L.A., Taubman, J., Greenberg, B.D., Xu, K., Arnold, P.D., Richter, M.A., Kennedy, J.L., Murphy, D.L., Goldman, D., 2006. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. Am. J. Hum. Genet. 78 (5), 815–826.

Hummerich, R., Reischl, G., Ehrlichmann, W., Machulla, H.J., Heinz, A., Schloss, P., 2004. DASB -in vitro binding characteristics on human recombinant monoamine transporters with regard to its potential as positron emission tomography (PET) tracer. J. Neurochem. 90 (5), 1218–1226.

Ichise, M., Liow, J.S., Lu, J.Q., Takano, A., Model, K., Toyama, H., Suhara, T., Suzuki, K., Innis, R.B., Carson, R.E., 2003. Linearized reference tissue parametric imaging methods: application to [11C]DASB positron emission tomography studies of the serotonin transporter in human brain. J. Cereb. Blood Flow Metab. 23 (9), 1096–1112.

Innis, R.B., Cunningham, V.J., Delforge, J., Fujita, M., Gjedde, A., Gunn, R.N., Holden, J., Houle, S., Huang, S.C., Ichise, M., Iida, H., Ito, H., Kimura, Y., Koeppe, R.A., Knudsen, G.M., Knuuti, J., Lammertsma, A.A., Laruelle, M., Logan, J., Maguire, R.P., Mintun, M.A., Morris, E.D., Parsey, R., Price, J.C., Slifstein, M., Sossi, V., Suhara, T., Votaw, J.R., Wong, D.F., Carson, R.E., 2007. Consensus nomenclature for in vivo imaging of reversibly binding radioligands. J. Cereb. Blood Flow Metab. 27 (9), 1533–1539.

Jacobsen, L.K., Staley, J.K., Zoghbi, S., Seibyl, J.P., Kosten, T.R., Innis, R.B., Gelernter, J., 2000. Prediction of dopamine transporter binding availability by genotype: a preliminary report. Am. J. Psychiatry 157 (10), 1700–1703.

Kalbitzer, J., Erritzoe, D., Holst, K.K., Nielsen, F.A., Marner, L., Lehel, S., Arentzen, T., Jernigan, T.L., Knudsen, G.M., 2010. Seasonal changes in brain serotonin transporter binding in short serotonin transporter linked polymorphic region-allele carriers but not in long-allele homozygotes. Biol. Psychiatry 67 (11), 1033–1039.

Kobiella, A., Reimold, M., Ulshöfer, D.E., Ikonomidou, V.N., Vollmert, C., Vollstädt-Klein, S., Rietschel, M., Reischl, G., Heinz, A., Smolka, M.N., 2011. How the serotonin transporter gene (5-HTTLPR) influences amygdala function: the role of in vivo serotonin transporter expression and amygdala structure. Transl. Psychiatry 1 (e37). doi:10.1038/tp.2011.29.

Lesch, K.P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274 (5292), 1527–1531.

Lesch, K.P., Waider, J., 2012. Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron 76 (1), 175–191.

Murthy, N.V., Selvaraj, S., Cowen, P.J., Bhagwagar, Z., Riedel, W.J., Peers, P., Kennedy, J.L., Sahakian, B.J., Laruelle, M.A., Rabiner, E.A., Grasby, P.M., 2010. Serotonin transporter polymorphisms (SLC6A4 insertion/deletion and rs25531) do not affect the availability of 5-HTT to [11C] DASB binding in the living human brain. Neuroimage 52 (1), 50–54.

Ohmoto, M., Hirakoshi, M., Mitsumoto, Y., 2013. Effects of moderating factors including serotonin transporter polymorphisms on smoking behavior: a systematic review and meta-analysis update. Nicotine Tob. Res. 15 (2), 572–582.

Olausson, P., Engel, J.A., Soderpalm, B., 2002. Involvement of serotonin in nicotine dependence: processes relevant to positive and negative regulation of drug intake. Pharmacol. Biochem. Behav. 71 (4), 757–771.

Parsey, R.V., Hastings, R.S., Oquendo, M.A., Huang, Y.Y., Simpson, N., Arcement, J., Huang, Y., Ogden, R.T., Van Heertum, R.L., Arango, V., Mann, J.J., 2006. Lower serotonin transporter binding potential in the human brain during major depressive episodes. Am. J. Psychiatry 163 (1), 52–58.

Philibert, R.A., Beach, S.R., Gunter, T.D., Brody, G.H., Madan, A., Gerrard, M., 2010. The effect of smoking on MAOA promoter methylation in DNA prepared from lymphoblasts and whole blood. Am. J. Med. Genet. B Neuropsychiatr. Genet. 153B (2), 619–628.

Philibert, R.A., Sandhu, H., Hollenbeck, N., Gunter, T., Adams, W., Madan, A., 2008. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa adoption studies. Am. J. Med. Genet. B Neuropsychiatr. Genet. 147B (5), 543–549.

Picciotto, M.R, Brunzell, D.H., Caldarone, B.J., 2002. Effect of nicotine and nicotinic receptors on anxiety and depression. Neuroreport 13 (9), 1097–1106.

Praschak-Rieder, N., Kennedy, J., Wilson, A.A., Hussey, D., Boovariwala, A., Willeit, M., Ginovart, N., Tharmalingam, S., Maselis, M., Houle, S., Meyer, J.H., 2007. Novel 5-HTTLPR allele associates with higher serotonin transporter binding in putamen: a [(11)C] DASB positron emission tomography study. Biol. Psychiatry 62 (4), 327–331.

Praschak-Rieder, N., Willeit, M., Wilson, A.A., Houle, S., Meyer, J.H., 2008. Seasonal variation in human brain serotonin transporter binding. Arch. Gen. Psychiatry 65 (9), 1072–1078.

Rausch, J.L., Fefferman, M., Ladisich-Rogers, D.G., Menard, M., 1989. Effect of nicotine on human blood platelet serotonin uptake and efflux. Prog. Neuropsychopharmacol. Biol. Psychiatry 13 (6), 907–916.

Reimold, M., Batra, A., Knobel, A., Smolka, M.N., Zimmer, A., Mann, K., Solbach, C., Reischl, G., Schwarzler, F., Gründer, G., Machulla, H.J., Bares, R., Heinz, A., 2008. Anxiety is associated with reduced central serotonin transporter availability in unmedicated patients with unipolar major depression: a [11C]DASB PET study. Mol. Psychiatry 13 (6), 606–613.

Reimold, M., Knobel, A., Rapp, M.A., Batra, A., Wiedemann, K., Ströhle, A., Zimmer, A., Schönknecht, P., Smolka, M.N., Weinberger, D.R., Goldman, D., Machulla, H.J., Bares, R., Heinz, A., 2011. Central serotonin transporter levels are associated with stress hormone response and anxiety. Psychopharmacology 213 (2–3), 563–572.

Reimold, M., Smolka, M.N., Schumann, G., Zimmer, A., Wrase, J., Mann, K., Hu, X.Z., Goldman, D., Reischl, G., Solbach, C., Machulla, H.J., Bares, R., Heinz, A., 2007a. Midbrain serotonin transporter binding potential measured with [(11)C]DASB is affected by serotonin transporter genotype. J. Neural Transm. 114 (5), 635–639.

Reimold, M., Smolka, M.N., Zimmer, A., Batra, A., Knobel, A., Solbach, C., Mundt, A., Smoltczyk, H.U., Goldman, D., Mann, K., Reischl, G., Machulla, H.J., Bares, R., Heinz, A., 2007b. Reduced availability of serotonin transporters in obsessive-compulsive disorder correlates with symptom sever. J. Neural Transm. 114 (12), 1603–1609.

Ribeiro, E.B., Bettiker, R.L., Bogdanov, M., Wurtman, R.J., 1993. Effects of systemic nicotine on serotonin release in rat brain. Brain Res. 621 (2), 311–318.

Ruhe, H.G., Booij, J., Reitsma, J.B., Schene, A.H., 2009. Serotonin transporter binding with [123I]beta-CIT SPECT in major depressive disorder versus controls: effect of season and gender. Eur. J. Nucl. Med. Mol. Imaging 36 (5), 841–849.

Shioe, K., Ichimiya, T., Suhara, T., Takano, A., Sudo, Y., Yasuno, F., Hirano, M., Shinohara, M., Kagami, M., Okubo, Y., Nankai, M., Kanba, S., 2003. No association between genotype of the promoter region of serotonin transporter gene and serotonin transporter binding in human brain measured by PET. Synapse 48 (4), 184–188.

Shumay, E., Logan, J., Volkow, N.D., Fowler, J.S., 2012. Evidence that the methylation state of the monoamine oxidase: a (MAOA) gene predicts brain activity of MAOA enzyme in healthy men. Epigenetics 7 (10), 1151–1160.

Staley, J.K., Krishnan-Sarin, S., Zoghbi, S., Tamagnan, G., Fujita, M., Seibyl, J.P., Maciejewski, P.K., O'Malley, S., Innis, R.B., 2001. Sex differences in [123I]beta-CIT SPECT measures of dopamine and serotonin transporter availability in healthy smokers and nonsmokers. Synapse 41 (4), 275–284.

van Dyck, C.H., Malison, R.T., Staley, J.K., Jacobsen, L.K., Seibyl, J.P., Laruelle, M., Baldwin, R.M., Innis, R.B., Gelernter, J., 2004. Central serotonin transporter availability measured with [123I]beta-CIT SPECT in relation to serotonin transporter genotype. Am. J. Psychiatry 161 (3), 525–531.

Vijayendran, M., Beach, S.R., Plume, J.M., Brody, G.H., Philibert, R.A., 2012. Effects of genotype and child abuse on DNA methylation and gene expression at the serotonin transporter. Front. Psychiatry 3, 55.

Wang, D., Szyf, M., Benkelfat, C., Provencal, N., Turecki, G., Caramaschi, D., Cote, S.M., Vitaro, F., Tremblay, R.E., Booij, L., 2012. Peripheral SLC6A4 DNA methylation is associated with in vivo measures of human brain serotonin synthesis and childhood physical aggression. PLoS One 7 (6), e39501.

Wendland, J.R., Martin, B.J., Kruse, M.R., Lesch, K.P., Murphy, D.L., 2006. Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531. Mol. Psychiatry 11 (3), 224–226.

Willeit, M., Stastny, J., Pirker, W., Praschak-Rieder, N., Neumeister, A., Asenbaum, S., Tauscher, J., Fuchs, K., Sieghart, W., Hornik, K., Aschauer, H.N., Brucke, T., Kasper, S., 2001. No evidence for in vivo regulation of midbrain serotonin transporter availability by serotonin transporter promoter gene polymorphism. Biol. Psychiatry 50 (1), 8–12.

Zhao, J., Goldberg, J., Bremner, J.D., Vaccarino, V., 2013. Association between promoter methylation of serotonin transporter gene and depressive symptoms: a monozygotic twin study. Psychosom. Med. 75 (6), 523–529.