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DNA methylation levels of the serotonin transporter gene are not associated with the outcome of highly standardized one-session exposure-based fear treatment

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ABSTRACT

Epigenetic alterations are regarded as a potential mechanism mediating the effects of environmental risk factors on vulnerability for a range of mental health problems. Recent studies have addressed the question whether DNA methylation patterns predict the outcome of psychological interventions and whether treatment effects might be associated with changes of DNA methylation. We assessed phobic fear symptoms, treatment-relevant traits and treatment response in 308 adults free of psychotropic medication - highly fearful of either spiders, blood-injury-injections, dental-treatments or heights - all subjected to highly standardized exposure-based one-session fear treatment. DNA methylation level of the promotor region of the serotonin transporter gene (*SLC6A4*) was assessed in either saliva samples (spider and dental treatment fear cohorts) or oral mucosa (BII, heights) to check whether possible effects are independent of the surrogate tissue examined. Moreover, in order to examine possible *DNA methylation* by *genotype* effects, patients were assessed for genetic variation of the serotonin transporter-linked polymorphic region (5-HTTLPR). DNA methylation levels were neither associated with pre-treatment fear levels, treatment relevant traits or treatment outcome data even when allelic variation of the 5HTTLPR was considered. Overall DNA methylation levels were higher in saliva samples compared to buccal samples. In saliva samples there was a small pre- to post-treatment increase in DNA methylation, which, however, was also not associated with the investigated phenotypes. We conclude that DNA methylation of *SLC6A4* is no suitable biomarker for response efficacy to highly standardized one-session exposure-based fear treatments.

1. Introduction

DNA methylation is considered a potential epigenetic candidate mechanism mediating the influence of environmental conditions on bio-behavioral reactivity to adversity (Ellis et al., 2011) and therefore increasingly investigated in relation to stress-related disorders (see Klengel and Binder, 2015; Peña et al., 2014; Nestler et al., 2016 for reviews). Initially, this view was supported by animal research demonstrating that the level of maternal care was associated with DNA methylation and expression of the glucocorticoid receptor gene in the rat's hippocampus, in turn leading to differences in stress regulation (Meaney, 2001; Weaver et al., 2004). Correspondingly, in humans, familial or social adversity such as childhood abuse, endured abusive parenting or peer-bullying has repeatedly been associated with altered DNA methylation patterns of genes involved in stress regulation and anxiety, including the serotonin transporter gene (*SLC6A4,* syn. 5-HTT) (Beach et al., 2010; Philibert et al., 2007). Moreover, it has been demonstrated that higher levels of *SLC6A4*-promotor methylation were associated with an increased reactivity to traumatic stress when accounting for the common serotonin transporter-linked polymorphism (5-HTTLPR) (Van Ijzendoorn et al., 2010), which is subdivided into S (short) lesser expressing and L (long) greater expressing alleles based on the presence of a 43 base pair repeat.

If DNA methylation mediates the effects of environmental conditions on mental health outcomes, then this might also be the case for the response to psychological treatments. Particularly, this may be promising in case of exposure-based approaches where inhibitory fear learning and its memory retrieval are postulated to represent key

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mechanisms for successful anxiety treatments (see Craske et al., 2014). On the neuronal level these two processes find correspondence in altered neuronal plasticity (see Margraf and Zlomuzica, 2015) which in turn on a cellular level likely are mediated via changes in gene expression and in the final consequence possibly controlled by epigenetic processes (see Kandel 2001; Kumsta 2019). DNA methylation levels assessed in accessible tissue such as blood, saliva, or oral mucosa might not necessarily reflect DNA methylation in brain tissue but might still serve as valuable biomarkers (Mill and Heijmans, 2013).

So far existing studies in search for DNA methylation as an epigenetic correlate or marker of treatment outcome in candidate genes (Knoblich et al., 2018; Perroud et al., 2013; Roberts et al., 2014, 2015, 2018; Yehuda et al., 2013; Ziegler et al., 2016) are highly heterogeneous in terms of the investigated samples, psychopathology, selected candidate genes, type of intervention and biochemical analyses of DNA methylation. As most of them report differential effects in responders and non-responders, although not always consistent regarding the direction of effects, they suggest pre-treatment DNA methylation and treatment-related DNA methylation change might be markers of treatment outcome. Only one study (Roberts et al., 2014) investigated DNA methylation change of *SLC6A4* in the context of psychological treatment so far. In children receiving cognitive-behavioral treatment (CBT) for an anxiety disorder, *SLC6A4* DNA methylation increased in treatment responders and decreased in non-responders mainly due to DNA methylation changes of one specific CpG-site.

Given the potential influence of the 5-HTTLPR on *SLC6A4* expression (Lesch et al., 1996), and given the association between the 5-HTTLPR and anxiety related phenotypes (although not supported by more recent work), it is conceivable that genetic variation of the *SLC6A4* additionally moderates any treatment effects on DNA methylation levels. However, to the best of our knowledge no study exists so far that had taken into account both *SLC6A4* methylation and 5-HTTLPR genetic variant in the context of psychological treatment.

In this study, a cohort of 308 adults highly fearful of either spiders, blood-injury-injections (BII), dental surgeries or heights was enrolled and subjected to highly standardized one-session exposure-based fear treatments. All participants were investigated for their DNA methylation of 5 CpGs close to the *SLC6A4* transcription start site (CpG −200; CpG −189; CpG −174; CpG −158; CpG −153) before and after treatment. In the spider fear and dental fear cohorts, which were treated temporally before the BII and fear of heights cohorts, saliva was used as a surrogate tissue. With the aim of using a more homogeneous tissue in terms of cell composition, buccal mucosa gained via swabs was used in the BII- and fear of heights cohorts. Even though in this sample the treated phobic fear and applied surrogate tissues are confounded within each fear cohort, differences in measured DNA methylation therefore could also be indicative of possible tissue type specific effects.

Our study pursued four main aims: First, we aimed to investigate whether pre-treatment DNA-methylation levels predict pre-treatment phobic fear severity and the manifestation of traits known to associate with the response to psychological treatment, such as trait-anxiety, depression and neuroticism (Min et al., 2012; Tyrer et al., 1992). Second, we aimed to test whether pre-treatment methylation moderates the outcome of treatment and whether the 5-HTTLPR genotype exerts a moderating role on the potential interplay between epigenome and the observed phenotypes.[1] Third, we explored whether the applied one session fear treatments associate with short-term changes in DNA-methylation and whether these changes are correlated with treatment response. Fourth, taking into account the described confounding between treated fear and tissue type the use of two different surrogate tissues may provide some insights on whether and how the results were influenced by the investigated surrogate tissue. Based on previous therapy epigenetic studies, we predict opposite direction of DNA methylation change in response to treatment.

2. Methods

The local Ethics Committee of the Faculty of Psychology, Ruhr University Bochum approved the study (Numbers 496R1; 453R1). An informed consent procedure was carried out with participants.

2.1. Participants and treatment

Participants aged between 18 and 70 years requested fear treatment at the Mental Health Research and Treatment Center in Bochum, Germany. They received detailed information on the treatment program for their respective fears on websites established for the project and registered for participation. The only inclusion criteria were subjective high and impairing fear of spiders, dental surgeries, BII or heights. In case of dental fear and BII fear, participants could self-screen their subjective fear by making use of a short questionnaire and were recommended to participate in the study if their scores indicated high fear or phobia, see (Wannemueller et al., 2016; 2017; 2018a; Wannemüller et al., 2019) for detailed descriptions.

Altogether, four treatment formats targeting the respective situational fear in the respective fear cohort were applied (spider fear, dental fear, BII-fear cohort, fear of heights cohort). Each treatment format was based on the recommendations for one-session treatments by Öst (1989), modified for use in large-group settings and delivered according to highly standardized treatment manuals (Wannemueller et al., 2016, 2017, 2018a; Wannemüller et al., 2019). In all treatment formats exposure was the main treatment component preceded by a psychoeducation phase, imparting information about the function of fear and aims of treatment. Furthermore, a video clip was presented, showing an expert in the respective field who deals with common misconceptions and myths about the feared stimuli. Exposure was delivered via video-clips and followed by live exposure elements in all conditions. Dental fear and BII fear interventions additionally contained the practice of bodily coping techniques to be applied during the exposure exercises. Exposure lasted for about 120 min in the spider fear and fear of heights treatment formats and 140 min in the dental and BII fear treatment formats. In all fear cohorts treatment formats were provided in a large group setting, where participants were gathered in an auditorium and treated simultaneously. Altogether in 249 participants (spider fear $n = 75$; dental fear $n = 39$; BII-fear $n = 40$; fear of heights $n = 95$) treatment was provided in a large-group setting. Furthermore, 59 individuals of the spider fear ($n = 24$) and BII fear ($n = 35$) cohorts were treated in an individual one-session format. During the individual one-session treatments, originally serving as control conditions to test for differences between individual and large-group one session treatments, the very same contents (video-clips etc.) as in the large group settings were administered by the same clinical psychologists for the same length (see Wannemueller et al., 2016, 2017 for more details).

As one-session treatments were applied in each case, the pre- and post-treatment epigenetic assessments took place at the same day. In all large-group conditions the pre-treatment methylation assessments were conducted at about 11 o'clock am. Post-treatment assessments were conducted between 4 and 6 o'clock pm.

2.2. Trait measures

The Trait-subscale of the State-Trait Anxiety Inventory (Spielberger et al., 1970) was used to assess trait-Anxiety. It ranges from 20 (no anxiety) to 80 (high anxiety) and the authors report very good retest-reliability (rtt = 0.96) and validity indices. We found an internal consistency (Cronbach's α) of $r = 0.92$ within our sample. Personality traits were assessed using the NEO-Five Factor Inventory (Costa and

[1] The effects of 5-HTTLPR genotype-variations on treatment response for parts of this cohort have already been reported in Wannemüller et al. (2018, 2018b).

McCrae, 1992) consisting of 60 items. The NEO-FFI is a well evaluated instrument and according to the authors showed sufficient reliability and validity. In our sample the internal consistency scores (Cronbach's α) ranged between 0.73 (agreeableness) and 0.87 (consciousness). We assessed subjective levels of symptom distress using the Depression-Anxiety-Stress Scale (DASS) (Lovibond and Lovibond, 1995) a well-established instrument with verified reliability and validity indices. In our sample we found a Cronbach's α = 0.91 for the DASS-total score.

2.3. Fear measures

We assessed post-treatment effects on subjective, cognitive and behavioral fear components using different fear questionnaire measures and a Behavioral Approach Test (BAT) in all four cohorts. In the spider-fear cohort we applied five questionnaire instruments and a 5-stepped BAT. In the BII-fear cohort, there were four questionnaire instruments and a 6-stepped BAT. In the dental-fear cohort again we used five questionnaires and an 8-stepped BAT. In the fear of heights cohort three questionnaires were used. Moreover, participants could ascend with a turntable ladder up to a maximum of 30 m (BAT). All questionnaire measures have been shown to validly and reliably assess the intended fear component (for a more detailed description on the applied instruments and reliability indices within the respective cohorts, see Wannemueller et al., 2016, 2017, 2018a; Wannemüller et al., 2019). Due to requirement of medical professionals, BATs could not be realized in the dental and BII conditions at follow-up assessment and one questionnaire in the dental fear cohort was not applied at follow-up. Therefore, they were excluded from analyses in the respective cohorts.

2.4. DNA extraction and 5-HTTLPR genotyping

In the spider-fear ($n = 99$) and dental-fear cohorts ($n = 39$) DNA was extracted from saliva samples using Oragene DNA (OG-500) kits. In the BII- ($n = 75$) and fear of heights ($n = 95$) cohorts, DNA was extracted from buccal swabs using the Masterpure DNA purification kit (Epicentre). Sample collection was realized following the protocols provided with the kits. The 43-base pair insertion/deletion of the 5-HTTLPR was genotyped using primers and PCR conditions as previously described (Wendland et al., 2006). Due to poor DNA quality, three individuals could not be genotyped for the 5-HTTLPR. The distribution of 5-HTTLPR genotypes did not differ from the Hardy-Weinberg criterion, $\chi 2 = .45$, $p = .57$.

2.5. DNA methylation

A total of 5 CpG sites located at the 5′ end of the *SLC6A4*-associated CpG island were examined for DNA methylation (chr17:28, 563, 138–28, 563, 186). DNA methylation of the spider fear, dental fear and blood, injury injection fear cohorts were analyzed by pyrosequencing; DNA methylation of the fear of heights cohort was analyzed by targeted-bisulfite sequencing (TBS; Moser et al., 2020). Sodium bisulfite conversion of 500 ng of genomic DNA from each participant was performed using the EZ DNA Methylation-GoldTM Kit (Zymo, USA).

In order to control for assay accuracies, *SLC6A4* assays were also performed using DNA methylation standards with a known degree of methylation (calibration mixtures of 0%, 50% and 100% methylated DNA). Results indicated no preferential DNA amplification in dependence of DNA methylation and results following a linear curve for all standards tested.

2.6. Data reduction

Concerning the one-session exposure treatments we calculated the following indices: 1. We defined the *pre-treatment fear* level as z-transformed mean score of all z-transformed pre-treatment scores of all questionnaire measures assessing the respective phobic fear and the BAT within the respective cohort. 2. We defined the *post-treatment fear reduction* as mean percentage pre to post change of all questionnaire fear measures and the BAT. 3. *Follow-up fear reduction* was calculated as described for post reduction using the follow-up scores instead. In order to calculate one indicator for *SLC6A4* promotor methylation and given substantial intercorrelations of methylation levels assessed at the five CpG sites ranging between $r = .14$ and $r = .55$ (all $p \leq .01$) a mean score of these assessments was built for each participant.

2.7. Statistical analyses

In order to investigate associations between DNA pre-treatment methylation and treatment relevant traits we used partial correlation analyses conducted in the whole sample adjusted for age and sex. Correlation analyses containing the 5-HTTLPR genotype were in addition either adjusted for tissue type or they were performed separately according to tissue type. Moreover, in order to investigate whether 5-HTTLPR and *SCL6A4*-methylation status interact to predict treatment outcome we calculated multiple linear regression analyses containing *SCL6A4* pre-methylation status, 5-HTTLPR genotype (homozygous l vs. homozygous s and heterozygous vs. homozygous l), the interaction between *SCL6A4*-methylation and 5-HTTLPR genotype, age, sex and tissue types with post-treatment fear reduction and follow-up fear reduction serving as dependent variables for the whole sample and both tissue types separately.

For DNA methylation change analyses, an rmANOVA of the form 2 (time) x 2 (tissue type) was calculated. Correlation analyses related to methylation changes were also either controlled for tissue type or performed separately. To prevent alpha error accumulation in repeated measurements, the significance level was decreased to $p < .01$ for the correlation analyses. All analyses were conducted using the IBM Statistics SPSS 27 software package.

3. Results

3.1. Sample description

SLC6A4 pre-treatment DNA methylation levels, fear levels, expression of treatment relevant traits as well as post-treatment and follow-up fear reductions for the whole sample and each cohort are described in Table 1. Cohorts differed in terms of their DNA methylation levels with largest DNA methylation levels assessed in the dental fear cohort and lowest levels in the fear of heights cohort. DNA methylation levels were higher in cohorts where saliva samples were used (dental fear and spider fear), $M = 3.04\ \% \pm .76\ \%$ compared to cohorts in which buccal cells were tested (BII fear, height fear), $M = 1.65\ \% \pm .91\ \%$, $F\ (1,307) = 208.90$, $p < .001$.

3.2. Associations between SLC6A4 pre-treatment methylation, fear levels and treatment-relevant traits in the whole cohort

SLC6A4 pre-treatment DNA methylation was not correlated with pre-treatment fear, treatment relevant traits or fear reduction at post- or follow-up assessments on a level of significance <.01, see Table 2. This was true for the whole sample and when analyzed separately in the respective treatment cohorts.

Regression analysis performed in the overall cohort showed a significant predictive effect for the interaction of *SCL6A4*-methylation and 5-HTTLPR genotype in predicting post-treatment outcome, as well as a highly significant effect of tissue type (and a highly significant age effect), see Table 3. Post-hoc correlation analyses conducted to follow-up the interaction effect show a significant correlation between *SCL6A4*-methylation and post-treatment fear reduction in the ss-genotype, $r = .348$, $p = .009$ and no associations in the other genotypes (ls: $r = .019$, $p = .81$; ll: $r = -.05$, $p = .61$).

Table 1
Description of the total sample and separated by treatment cohorts.

		Total (n = 308)	Spider fear (S) (n = 99)	BII fear (BII) (n = 75)	Dental fear (D) (n = 39)	Fear of heights (H) (n = 95)	Group comparison
Age		35.83 ± 13.83	32.22 ± 12.14	26.05 ± 7.09	51.10 ± 11.55	41.30 ± 12.54	BII < S < H < D
Sex	male	62	9	9	8	36	n.s.
	female	243	88	66	31 0	58	
	missing	3	2	0		1	
5-HTTLPR genotype[a,b]	***ll***	95	31	21	13	30	n.s.
	ls	155	52	37	20	46	
	ss	55	15	16	6	18	
SLC6A4-methylation							
Pre-treatment (%)		2.31 ± 1.06	2.90 ± 0.77	2.06 ± 0.63	3.42 ± 0.59	1.32 ± 0.96	H < BII < S < D
Post-treatment (%)		2.43 ± 1.22	3.17 ± 0.89	2.25 ± 0.58	3.74 ± 0.63	1.26 ± 1.00	H < BII < S < D
Trait Measures							
DASS-21 Depression[c]		3.56 ± 3.71	3.51 ± 4.01	–	4.84 ± 4.03	3.08 ± 3.13	H < D
DASS-21 Anxiety		3.55 ± 3.38	3.15 ± 3.08	–	4.59 ± 4.03	3.54 ± 3.36	n.s.
DASS-21 Stress		8.11 ± 4.82	8.21 ± 4.57	–	8.85 ± 4.62	7.69 ± 5.14	n.s.
NEO-Neuroticism		1.85 ± 0.69	1.84 ± 0.64	1.98 ± 0.66	1.94 ± 0.84	1.76 ± 0.69	n.s.
NEO-Extraversion		2.46 ± 0.56	2.47 ± 0.51	2.46 ± 0.69	2.41 ± 0.61	2.47 ± 0.55	n.s.
NEO-Openness		2.55 ± 0.58	2.51 ± 0.57	2.36 ± 0.55	2.52 ± 0.68	2.70 ± 0.53	BII < H
NEO-Agreeableness		2.69 ± 0.49	2.75 ± 0.48	2.70 ± 0.50	2.75 ± 0.45	2.59 ± 0.49	n.s.
NEO-Conscientiousness		2.75 ± 0.64	2.72 ± 0.66	2.89 ± 0.67	2.73 ± 0.55	2.75 ± 0.64	n.s.
Phobic fear							
Pre-treatment (z-score)		−0.02 ± 1.00	−0.03 ± 1.00	−0.01 ± 0.97	0.01 ± 1.01	−0.02 ± 1.02	n.s.
Post reduction (%)[d]		28.03 ± 19.49	37.12 ± 20.76	21.82 ± 18.05	17.53 ± 17.61	27.80 ± 15.92	BII, D < H < S
Follow-up reduction (%)[e]		26.31 ± 22.46	34.00 ± 26.29	24.95 ± 21.58	19.52 ± 20.39	26.27 ± 21.24	n.s.

Note:
[a] Missing information on 5-HTTLPR genotype due to insufficient DNA-quality for three participants.
[b] ll = long/long, ls = long/short, ss = short/short.
[c] The DASS-21 was not conducted in the BII-cohort.
[d] Due to premature drop-out ($n = 8$), data on post treatment *SLC6A4*-metylation and post treatment fear reduction was available in 300 participants.
[e] Data on follow-up fear reduction was available in $n = 152$ participants.

Table 2
Correlations between pre-treatment *SLC6A4* methylation, pre-treatment fear levels, treatment relevant traits and fear reduction.

	Whole sample					5-HTTLPR genotype[a]					
						ll		ls		ss	
	Total (n = 308)	Spider fear	BII fear	Dental fear	Fear of heights	saliva	buccal swabs	saliva	buccal swabs	saliva	buccal swabs
Pre-treatment fear (z-score)	−.02	.01	.10	−.03	−.11	−.08	.12	−.10	−.10	−.09	.10
Post-treatment reduction (%)[a]	.06	.13	−.02	−.23	.03	−.19	−.18	−.05	−.01	.30	−.05
Follow-up reduction (%)[b]	−.04	−.12	.05	.09	−.16	−.27	−.05	−.06	−.17	−.06	.16
Trait Measures											
DASS-Depression	−.01	−.21	–	.06	−.12	−.06	−.07	.08	.05	−.30	−.19
DASS-Anxiety	−.02	−.04	–	.11	−.09	−.14	.14	.27	−.11	−.20	.24
DASS-Stress	−.00	−.05	–	.28	−.18	−.06	.08	.15	−.08	−.12	−.07
NEO-Neuroticism	.02	.04	−.03	.14	−.18	.01	−.14	.15	−.11	.06	−.31
NEO-Extraversion	−.02	−.11	.12	.12	.00	−.16	.14	.01	−.05	−.10	.12
NEO-Openness	−.05	.14	−.13	.34	−.09	.09	−.17	.16	−.11	.58	−.45
NEO-Agreeableness	.11	−.11	.12	−.08	.-08	−.02	.29	−.22	.08	.03	−.08
NEO-Conscientiousness	.01	−.02	.13	−.09	.08	.12	.18	−.23	.06	.34	.26

Note. Correlation analyses were all adjusted for age, sex and pre-treatment fear level; none of the reported correlation coefficients reached the Bonferroni corrected level of significance, $p < .0045$.
[a] ll = long/long, ls = long/short, ss = short/short.

However, as can be seen in Fig. 1 the significant correlation in the ss-genotype was due to a confounding with tissue type, as methylation levels in fear cohorts assessed with saliva-samples (spider fear and dental fear) were larger compared to methylation levels assessed via buccal swabs (BII-fear and fear of heights). Two correlation analyses conducted separately by tissue type in the ss-genotype yielded neither in the saliva ($r = .137$, $p = .55$) nor buccal sample ($r = -.032$, $p = .85$) a significant correlation between *SCL6A4*-methylation and post-treatment fear reduction.

The influence of tissue type was confirmed by regression analyses conducted separately for both tissue types which no longer show any significant effects concerning the interaction of *SCL6A4*-methylation and 5-HTTLPR genotype, see Table 3.

3.3. Changes in DNA-methylation, interactions with 5-HTTLPR genotype and associations to treatment outcome

There were no significant correlations between DNA methylation change from pre-to post-treatment and post- or follow-up treatment outcome, see Table 4.

A 2 (time) x 2 (tissue type) x 3 (genotype) analysis showed a large main effect of tissue type, $F(1,298) = 269,71$ $p < .001$. In saliva samples

Table 3
Results of multiple linear regression analyses, predicting fear reduction post-treatment and at follow-up assessment containing 5-HTTLPR genotype, *SLC6A4*-methylation and their interaction.

	Whole Sample						Saliva						Buccal mucosa					
	Post-treatment			Follow-up			Post-treatment			Follow-up			Post-treatment			Follow-up		
	β	T	*p*	β	T	*p*	β	T	*p*	β	T	*p*	β	T	p	β	T	p
Genotype[1] (ll vs. ss)[a]	.357	1.962	.051	.372	1.55	.121	.451	.757	.450	.587	.597	.554	.195	.779	.437	.501	1.69	.093
Genotype[2] (ls vs. ls)	.314	1.677	.095	.242	.975	.331	.503	.799	.426	.307	.267	.791	.098	.386	.700	.563	1.88	.063
SCL6A4 pre-treatment methylation	.246	1.500	.135	−.090	−.393	.695	.300	1.07	.285	−.048	−.091	.928	−.059	−.242	.809	.205	.785	.435
Genotype[1] x *SCL6A4* pre-treatment methylation	−.455	−2.111	**.036**	.131	.613	.540	−.785	−1.23	.220	−.067	−.054	.957	.022	.069	.945	−.559	−1.50	.137
Genotype[2] x *SCL6A4* pre-treatment methylation	−.495	−2.482	**.014**	−.097	−.324	.747	−.721	−1.15	.250	−.354	−.323	.748	−.133	−.504	.615	−.311	−.915	.363
Age	−.185	−3.232	**.001**	−.075	−.891	.375	−.394	−4.74	**<.001**	.058	.380	.706	.005	.060	.952	−.081	−.770	.443
Sex	−.014	−.246	.806	−.058	−.707	.481	.040	.489	.626	.206	1.42	.162	−.084	−1.04	.299	−.202	−2.00	.048
Tissue type	−.226	−3.072	**.002**	−.158	−1.40	.162	–	–	–	–	–	–	–	–	–	–	–	–

Note: Significant results were bold printed.
[a] ll = long/long, ls = long/short, ss = short/short.

(spider fear and dental fear groups), a small pre-to post-treatment increase of $M = .28\ \% \pm .67\ \%$ in DNA-methylation was observed, compared to no change when buccal cells were analyzed ($M = .05\ \% \pm .97\ \%$; 2-way interaction time by tissue type, $F(1,298) = 7.30$, $p = .007$), see Fig. 2. No other main or interaction effect reached the level of significance, i.e., carriers of different 5-HTTLPR allelic variants neither differed concerning *SCL6A4*-methylation levels nor concerning pre-to post-treatment change in *SCL6A4*-methylation.

4. Discussion

In this *'therapyepigenetic'* study conducted in a sample of individuals with different phobic fears we investigated whether pre-treatment DNA methylation levels of *SLC6A4* were associated with pre-treatment severity of phobic fear, treatment-relevant traits such as neuroticism and treatment outcome, i.e., the decrease of phobic fear following a highly standardized exposure-based one-session fear treatment. Moreover, we aimed to investigate whether treatment outcome was associated with changes in DNA methylation levels and whether potential changes would associate with outcome data resulting from a 6-month follow-up assessment. As we further aimed to gain insights on whether and how possible epigenetic effects were influenced by genetic variation, we took account for the 5-HTTLPR genotypes in our analyses. As we used saliva and oral mucosa as surrogate tissues in two fear cohorts, we were able to investigate possible effects of the tissue type, taking into account the confounding of fear cohort and tissue type.

However, regardless of whether obtained from saliva samples (spider fear and dental fear cohort) in which overall methylation was higher than in buccal cells or obtained from the latter (BII-fear and fear of heights cohorts) pre-treatment *SLC6A4* DNA methylation levels were neither associated with pre-treatment levels of phobic fear nor with traits known to exert an adverse effect on treatment outcome such as depression, anxiety, stress or neuroticism. Moreover, there were no associations between pre-treatment *SLC6A4* DNA methylation levels and immediate as well as long-term treatment response. Null findings were also not influenced by variations in the 5-HTTLPR, although regression analysis in the whole sample showed a predictive effect for the interaction of *SCL6A4*-methylation and 5-HTTLPR genotype, due to a significant association between *SCL6A4*-methylation and post-treatment fear reduction in the ss genotype. As post-hoc analyses showed, however, this effect was not observed independently of tissue type and most likely arises from the fact that higher methylation levels existed in cohorts measured with saliva and were confounded with higher post-treatment fear reductions.

A similar picture emerged concerning the second main aim of our study which consisted in exploring changes in DNA methylation in the context of treatment. Again, results were related to tissue-type. Whereas there was no pre-to post-treatment change in oral mucosa samples we observed a small DNA methylation increase in the saliva samples, which however is most likely of no biological relevance (magnitude of change was below 1%). The observed minor differences can most likely be explained by shifts in cellular composition (see Kumsta, 2019 for detailed insights in underlying processes). Furthermore, change in DNA methylation was unrelated to symptom changes. Future '*therapyepigenetic*' trials should account for tissue-type effects (Hummel et al., 2021).

The following limitations of the study need to be considered. First, it did not include a control group so that there was no possibility to control our results for stochastic fluctuations in DNA-methylation. Second, albeit Pyrosequencing and targeted-bisulfite sequencing, as used here, are amongst the most sensitive and accurate applications to analyse DNA methylation, the observed changes of DNA-methylation from pre to post treatment were smaller than the assays sensitivity. Performing pyrosequencing in duplicates/triplicates would have verified reliability of measurements but unfortunately was not affordable. Third, as already mentioned, phobic fear and tissue type were confounded in our sample.

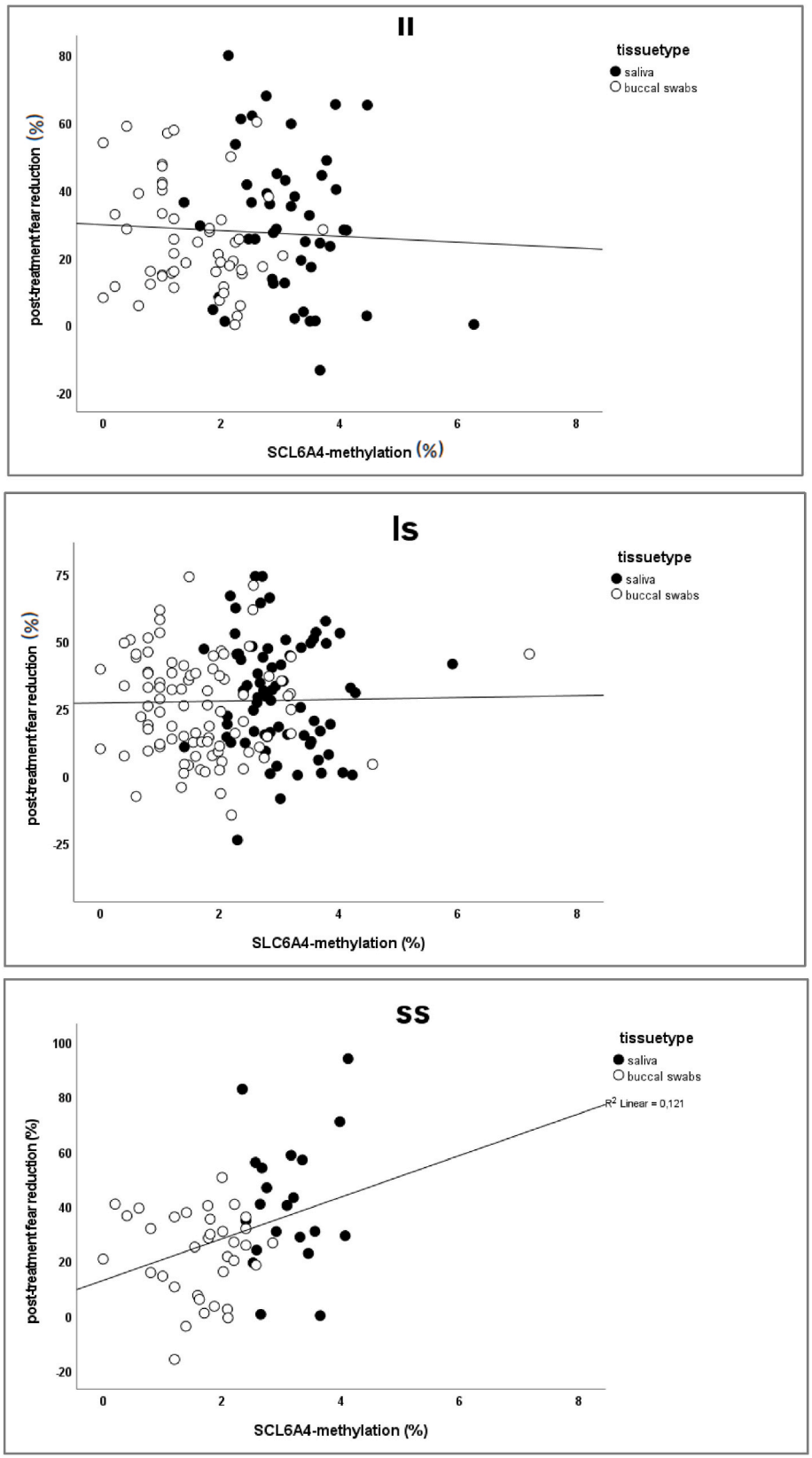

Fig. 1. Boxplots of the correlation between *SCL6A4*-methylation (%) and post-treatment fear reduction (%) in 5-HTTLPR genotypes.
Note: ll = long/long, ls = long/short, ss = short/short.

Table 4
Correlations between pre-to post-treatment changes in *SLC6A4*-methylation and fear reduction.

	Whole sample					5-HTTLPR genotype[a]		
	Total	Spider fear	BII fear (BII)	Dental fear	Height fear	ll	ls	ss
Post-reduction (%)	.01	−.01	−.05	−.14	.10	−.20	.10	.13
Follow-up reduction (%)	.04	.06	.10	−.30	−.02	−.16	.11	.48
Fear return (%)	.01	−.01	−.03	.21	−.11	−.14	.04	.26

Note: All correlations are adjusted for age, sex and pre-treatment fear level. Correlations conducted in the 5HTTLPR genotype groups were additionally adjusted for tissue type.
[a] ll = long/long, ls = long/short, ss = short/short.

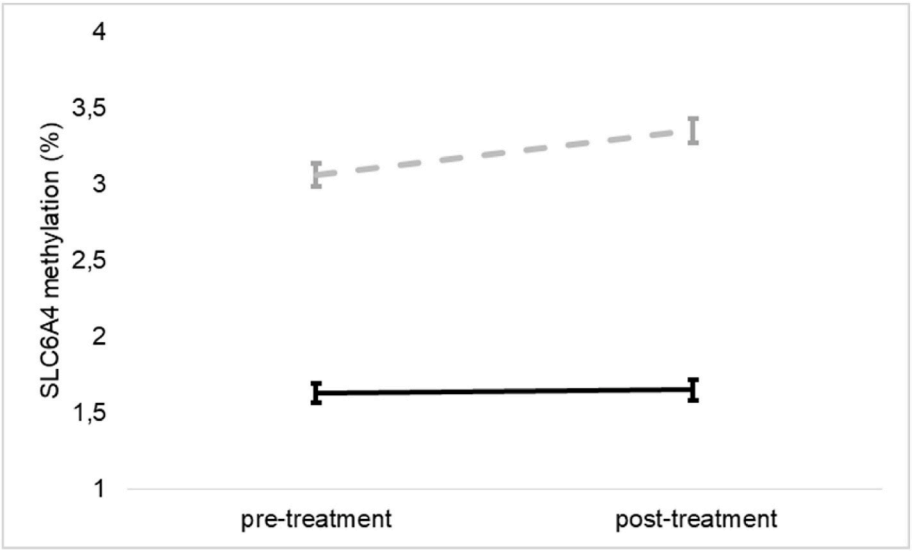

Fig. 2. Change in *SLC6A4*-metylation from pre-to post-treatment assessment following the one-session exposure-based fear treatments assessed in either saliva samples (grey dashed line) or buccal swabs (black line).
Note: error bars indicate standard errors.

Hence, albeit not very likely given that all participants suffered from situational phobic fears and considering the comparably large differences in *SCL6A4* pre-treatment methylation between cohorts in which different tissues were applied compared to much smaller differences between fear cohorts in which the same tissue was applied (for the exact figures see Table 1), it cannot be ruled out that the observed differences in *SCL6A4*-methylation that we referred to tissue-type effects, actually were due to the respective situational fears. Fourth, the same was true for the interaction between genotype and methylation, which significantly predicted post-treatment fear reduction due to a correlation between pre-treatment methylation and post-treatment fear reduction in homozygous S-allele carriers (see Table 3 and Fig. 1). However, the interaction was not predictive within cohort comparisons where the same tissue material was used. Moreover, within the ss genotype neither within saliva nor buccal samples pre-treatment methylation and fear reduction were associated. Hence, it is much likelier that the effect was due to differences in methylation levels of the different tissues types and a confounding with fear reductions (altogether there were larger fear reductions in cohorts were saliva was used) that contributed to the reported effect in the comparatively small group of homozygous S-allele carriers than driven by real epigenetic effects in the low-expressing genotype. However, only future studies that show a complete internal balance between fear type and tissue type could provide complete certainty here.

In conclusion, our results do not support genetic variation or DNA methylation of the serotonin transporter gene as a promising biomarker predicting or reflecting therapy outcome. The overall small variance and low levels of DNA methylation of the *SLC6A4* promoter-near region further dampen expectations for the serotonin transporter gene as a useful marker in the context of '*therapyepigenetic*' research. These findings support the growing doubts of further pursuing research involving the serotonin transporter gene in mental health research.

CRediT authorship contribution statement

André Wannemüller: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Robert Kumsta:** Conceptualization, Writing – review & editing. **Dirk Moser:** Methodology. **Hans-Peter Jöhren:** Data curation. **Jürgen Margraf:** Funding acquisition, Resources.

Declaration of competing interest

The authors report no biomedical financial interests or potential conflicts of interest.

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