

# Peripheral serotonin transporter DNA methylation is linked to increased salience network connectivity in females with anorexia nervosa

Ilka Boehm, PhD\*; Esther Walton, PhD\*; Nina Alexander, PhD;  
Victoria-Luise Batury, MSc; Maria Seidel, PhD; Daniel Geisler, MSc; Joseph A. King, PhD;  
Kerstin Weidner, MD; Veit Roessner, MD; Stefan Ehrlich, MD, PhD

**Background:** Epigenetic variation in the serotonin transporter gene (*SLC6A4*) has been shown to modulate the functioning of brain circuitry associated with the salience network and may heighten the risk for mental illness. This study is, to our knowledge, the first to test this epigenome–brain–behaviour pathway in patients with anorexia nervosa. **Methods:** We obtained resting-state functional connectivity (rsFC) data and blood samples from 55 acutely underweight female patients with anorexia nervosa and 55 age-matched female healthy controls. We decomposed imaging data using independent component analysis. We used bisulfite pyrosequencing to analyze blood DNA methylation within the promoter region of *SLC6A4*. We then explored salience network rsFC patterns in the group × methylation interaction. **Results:** We identified a positive relationship between *SLC6A4* methylation levels and rsFC between the dorsolateral prefrontal cortex and the salience network in patients with anorexia nervosa compared to healthy controls. Increased rsFC in the salience network mediated the link between *SLC6A4* methylation and eating disorder symptoms in patients with anorexia nervosa. We confirmed findings of rsFC alterations for CpG-specific methylation at a locus with evidence of methylation correspondence between brain and blood tissue. **Limitations:** This study was cross-sectional in nature, the sample size was modest for the method and methylation levels were measured peripherally, so findings cannot be fully generalized to brain tissue. **Conclusion:** This study sheds light on the neurobiological process of how epigenetic variation in the *SLC6A4* gene may relate to rsFC in the salience network that is linked to psychopathology in anorexia nervosa.

## Introduction

The serotonin system has been associated with mood regulation, anxiety and the modulation of appetite,<sup>1</sup> all of which nominate this neurotransmitter system as a potential candidate for involvement in the pathogenesis of anorexia nervosa.<sup>2</sup> Anorexia nervosa is characterized by severe food restriction and affective symptoms (such as depressed mood and difficulties in emotion regulation),<sup>3</sup> as well as a heightened prevalence of anxious and obsessive personality traits.<sup>4</sup> A number of studies have suggested reduced serotonergic tone during the acute undernourished state of the disorder: for example the serotonin metabolite 5-hydroxyindolacetic acid in cerebrospinal fluid<sup>5</sup> was decreased, plasma prolactin response to serotonin agonists were blunted,<sup>6</sup> and whole blood serotonin content and tryptophan levels<sup>7</sup> were re-

duced. However, patients with anorexia nervosa in the weight-recovered state may exhibit hyperserotonergic dysfunction.<sup>8</sup> The assumption that increased serotonin transmission is a trait marker of the disorder is supported by findings of decreased platelet monoamine oxidase B activity<sup>9</sup> and altered cerebral serotonin 1A and 2A receptor binding.<sup>10,11</sup> It has been hypothesized that serotonergic dysfunctions may drive body-image distortions and self-starvation in anorexia nervosa.<sup>12</sup>

However, associations between genetic polymorphisms of the serotonergic system (in particular the serotonin transporter *SLC6A4*) and anorexia nervosa could not be replicated.<sup>13</sup> Therefore, an interest in the epigenome has gained momentum. Epigenetic processes such as DNA methylation may mediate environmental effects on gene expression without changing the DNA sequence. During DNA methylation, a methyl group is

**Correspondence to:** S. Ehrlich, Division of Psychological and Social Medicine and Developmental Neurosciences, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany; Translational Developmental Neuroscience Section, Fetscherstrasse 74, 01307 Dresden, Germany; stefan.ehrlich@uniklinikum-dresden.de

\*These authors contributed equally to this work.

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added to a cytosine residue, often adjacent to a guanine nucleotide. These so-called CpG sites tend to cluster together in CpG islands. In the promoter region of genes, CpG islands are often demethylated, whereas the methylated state is commonly associated with reduced gene expression.<sup>14</sup> Within the *SLC6A4* promoter region, DNA methylation appears to be of functional relevance, because it has been found to predict lower *SLC6A4* mRNA expression.<sup>15</sup> Furthermore, we previously reported that DNA methylation at CpG site 13 (cg14692377) showed a significant correlation between blood and brain tissue obtained simultaneously from paired blood and temporal lobe biopsy samples.<sup>16</sup> This CpG was also shown to be correlated in paired pre-mortem blood and post-mortem brain samples.<sup>17</sup> However, although *SLC6A4* methylation has been reported to play a role in depressive disorders and schizophrenia,<sup>18</sup> the only existing candidate-gene study in anorexia nervosa found no group differences compared to healthy controls.<sup>19</sup> As well, a study using a methylome-wide approach did not provide support for a link between *SLC6A4* DNA methylation and a diagnosis of anorexia nervosa.<sup>20</sup> However, related phenotypes such as cortisol stress reactivity have been repeatedly associated with altered *SLC6A4* methylation.<sup>21</sup> Expanding the scope by integrating epigenetic and neuroimaging data may allow us to test the complex interplay between genes, brain function and abnormal behaviour related to eating disorders.<sup>22,23</sup>

One promising neuroimaging technique is resting-state functional connectivity (rsFC), which is defined as the temporally correlated, low-frequency, blood oxygen level-dependent (BOLD) signal fluctuation of distinct brain regions while the participant is at rest. This method is sensitive to changes in communication between spatially separated brain regions and is thought to be more sensitive to the effects of genetic and environmental variation in heterogeneous phenotypes such as psychiatric disorders.<sup>24,25</sup>

Recent evidence from rsFC studies points to a dysfunction in the salience network as a prominent feature of several mental disorders,<sup>24</sup> including anorexia nervosa. The salience network — consisting of the dorsal anterior cingulate cortex, the insular cortices and additional limbic structures such as the amygdala<sup>24</sup> — is associated with detecting, filtering and integrating biologically relevant sources of salience, such as emotional information, homeostatic regulation and reward value.<sup>26</sup> Some studies have demonstrated aberrant functional within- and between-network connectivity of the salience network in anorexia nervosa, but others found only indirect evidence for salience network abnormalities in patients in the acutely underweight state.<sup>27,28</sup> Importantly, studies in healthy participants have observed that activity in key nodes of the salience network — including the amygdala<sup>29</sup> and insula,<sup>30</sup> as well as amygdala connectivity<sup>31</sup> — might depend on *SLC6A4* methylation. Using a monozygotic twin design, a recent study demonstrated an association between *SLC6A4* methylation and brain responses to negative stimuli in frontal-limbic regions overlapping the salience network that was independent of DNA sequence variations.<sup>32</sup>

Given that previous studies in anorexia nervosa have identified neural alterations in the salience network, and that this network has also been identified as a neural correlate of

*SLC6A4* methylation, we assumed that a serotonin-related epigenome–brain–behaviour process, discussed in detail in a study by Palma-Gaudiel and Fañanás,<sup>18</sup> could also be highly relevant for anorexia nervosa. These authors describe a neurobiological pathway that is rooted in a reduction of serotonin transporter expression mediated by *SLC6A4* methylation. This reduction may lead to changes in serotonergic tone, associated with altered reactivity and connectivity of brain regions and mental health problems relevant to the salience network.

In light of the proposed epigenome–brain–behaviour pathway for anorexia nervosa, the aim of this study was to examine the associations between *SLC6A4* methylation, salience network rsFC and eating disorder symptoms in acutely ill patients with anorexia nervosa compared to matched healthy controls.

## Methods

### Participants

Our sample consisted of 110 female volunteers: 55 participants with acute anorexia nervosa according to the *Diagnostic and Statistical Manual of Mental Disorders, 5th edition* (DSM-5; age 12–28 yr) and 55 pair-wise age-matched female healthy controls (age 12–28 yr). All patients with anorexia nervosa were assessed within 96 hours after the beginning of a behaviourally oriented nutritional rehabilitation program. In the anorexia nervosa group, 53 of the patients were of the restrictive and 2 of the binge/purge subtype; 7 had comorbid psychiatric disorders (4 patients with depressive disorders including dysthymia, 3 with anxiety disorder, and 1 with obsessive–compulsive disorder). Six patients with anorexia nervosa and 10 healthy controls reported that they smoked cigarettes currently or had smoked in the past.

We applied several exclusion criteria for each group (Appendix 1, Supplemental Materials 1.1, available at [jpn.ca/190015-a1](http://jpn.ca/190015-a1)), most importantly psychotropic medication other than selective serotonin reuptake inhibitors (SSRIs) within 4 weeks before the study ( $n = 1$ ), binge eating or diagnosis of bulimia nervosa, substance abuse, or neurologic or medical conditions. This study was approved by the review board of the Technische Universität Dresden, and all participants (and, if under-age, their guardians) gave written informed consent.

### Clinical measures

To ascertain the absence or presence of a current eating disorder, we administered the expert form of the Structured Interview for Anorexia and Bulimia Nervosa for DSM-IV (SIAB-EX<sup>33</sup>) in all participants. Interviews were adapted to DSM-5 criteria (no amenorrhea criteria) and carried out by clinically experienced and trained research assistants under the supervision of the attending child and adolescent psychiatrist. We estimated intelligence quotient using a short version of the German adaptation of the Wechsler Adult Intelligence Scale,<sup>34</sup> or a short version of the German adaptation of the Wechsler Intelligence Scale for Children<sup>35</sup> for participants aged 15 years or younger.

We assessed eating disorder-specific psychopathology using the short version of the Eating Disorders Inventory (EDI-2).<sup>36</sup> We examined expressive symptoms using the German version of the Beck Depression Inventory (BDI-II).<sup>37</sup> We investigated symptoms of anxiety using the anxiety subscale of the Symptom Checklist 90 Revised (SCL-90-R).<sup>38</sup>

#### *Blood DNA methylation, sample processing and quality control*

Quantitative peripheral DNA methylation at 15 CpG sites in the promoter-associated CpG island of *SLC6A4* initially described by Philibert and colleagues<sup>39</sup> was performed by Varionostic GmbH ([www.varionostic.de](http://www.varionostic.de)). We focused on 15 CpG sites within amplicon 3 of a 799 bp region (originally CpG 43–57, referred to in the current study as CpG 1–15), because a previous study observed significant interindividual variation in methylation in this subregion (but not in others) and found a significant correlation with rsFC in the salience network.<sup>31</sup> As well, DNA methylation in this subregion has been shown to be correlated between blood and brain tissue.<sup>16</sup> Moreover, previous studies have argued that CpG-specific methylation (rather than average methylation) corresponds with mRNA expression.<sup>15,40</sup> To further investigate the correlation between peripheral, blood-based measures of *SLC6A4* DNA methylation with those in postmortem brain tissue, we queried BECon, an online database of cross-tissue DNA methylation.<sup>41</sup> Based on whole-blood and postmortem brain tissue from 3 regions (frontal, temporal and parietal) obtained from 16 individuals, cg14692377 showed moderate-rank correlations between tissues ( $\rho$  BA10 = 0.29 [frontal];  $\rho$  BA20 = 0.36 [temporal];  $\rho$  BA10 = 0.48 [parietal]), indicating that in this specific case peripheral DNA methylation might indeed index brain-based methylation patterns. For analysis, genomic DNA was extracted from EDTA whole-blood samples and bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research). Subsequent pyrosequencing was performed on the Q24/ID System, and percent DNA methylation at each CpG site was quantified using the PyroMark Q24 software (Qiagen). For a detailed protocol containing amplicon, sequencing, and cross-CpG correlations and transcription factor binding site within the region, see Appendix 1, Supplemental Materials 1.2. We visually inspected pyrograms to individually verify DNA methylation estimates if they were at least 3 standard deviations away from the mean, or if internal quality scores indicated a deviation from the expected pattern. Based on these criteria, we excluded data from 1 CpG (CpG 15). The mean missingness after exclusion was 0.58% across CpGs (range: 0%–1.82%) and participants (range: 0%–35.71%). Missing values were imputed using predictive mean matching with default settings as implemented in the R mice package.<sup>42</sup>

#### *MRI data acquisition and preprocessing*

Data were acquired with a 3T Siemens Trio. We acquired the  $T_1$ -weighted structural brain scans with a rapid acquisition gradient-echo (MP-RAGE) sequence (parameters are described

in Appendix 1, Supplemental Materials 1.3). We acquired an 8-minute resting functional MRI (fMRI) scan using gradient-echo  $T_2^*$ -weighted echo planar imaging with standard parameters (Appendix 1, Supplemental Materials 1.3). During fMRI, participants were instructed to lie still with closed eyes and not fall asleep. Functional and structural images were processed using SPM8 ([www.fil.ion.ucl.ac.uk/spm/](http://www.fil.ion.ucl.ac.uk/spm/)) within the Nipype framework (<http://nipy.sourceforge.net/nipype>) following standard procedures (Appendix 1, Supplemental Materials 1.4).

We evaluated the quality of the fMRI data using manual inspection and artifact detection tools (Appendix 1, Supplemental Materials 1.4).

#### *Independent component analysis and identification of component of interest*

To extract temporally coherent networks that reflected well-defined circumscribed functional properties, we conducted a spatial group independent component analysis (ICA)<sup>43</sup> for all participants using the Group ICA fMRI Toolbox (GIFT) implemented in Matlab (<http://mialab.mrn.org/software/gift>). Unlike seed-based approaches, this technique allows for identification of a resting-state network that is independent of neuroanatomical variability. After initial data reduction using principal component analysis, we decomposed the fMRI data into 24 maximally independent components by applying the infomax algorithm (Appendix 1, Supplemental Materials 1.5). For each participant, we back-reconstructed component spatial maps using GICA and converted to  $z$  values.<sup>44</sup> As in our previous reports,<sup>45,46</sup> we identified components of interest by spatial correlation with the relevant templates (salience, frontoparietal and visual network) from a study by Yeo and colleagues.<sup>47</sup> We mapped independent component numbers 9 (IC9) and 13 (IC13) onto the salience network template defined by Yeo and colleagues<sup>47</sup> (Appendix 1, Supplemental Materials 1.6).

#### *Statistical models*

We averaged DNA methylation levels across the 14 individual CpG sites after quality control and imputation to calculate a mean *SLC6A4* methylation score (methylation<sub>mean</sub>) for each participant.<sup>31</sup> Although *SLC6A4* methylation<sub>mean</sub> was our main measure of interest, we also investigated the effects of CpG site 13 (methylation<sub>CpG13</sub>), because there is evidence<sup>16</sup> for small to moderate blood–brain correspondence of methylation<sub>CpG13</sub>. To identify the effects of potentially confounding variables on DNA methylation, we investigated associations with age (using Pearson  $r$ ).

For rsFC, we entered spatial maps of the components of each participant into SPM8 to conduct the following analyses. First, to test for basic group differences, we performed a 2-sample  $t$  test for the 2 components identified as salience network (step 1).<sup>47</sup> Second, we examined components of the main model for which we had found a basic group difference at step 1. The rsFC in components that showed a group difference in step 1 were regressed using group, *SLC6A4* methylation<sub>mean</sub>,

their interaction, and age as covariates; the interaction term was our main effect of interest (step 2). Third, based on previous findings as outlined earlier<sup>15,16</sup> we reran the main model described in step 2 using methylation<sub>CpG13</sub> instead of methylation<sub>mean</sub> (step 3). Fourth, to investigate the specificity of the effect of DNA methylation on the rsFC of the salience network, we examined the group  $\times$  methylation<sub>mean</sub> interaction with a control analysis for the visual network (also identified by spatial correlation with a template<sup>47</sup>), a brain network that is thought to be somewhat less influenced by the serotonergic system<sup>48</sup> (step 4). The results of all models were masked using the aforementioned RSN templates<sup>47</sup> and had to exceed  $p < 0.05$  (family-wise error [FWE] correction) to guard against type I errors. To test whether our main analysis (step 2) was influenced by smoking, we ran additional subanalyses excluding participants who smoked currently or had smoked in the past (anorexia nervosa = 6; healthy controls = 10; including occasional smoking) or were taking SSRIs (anorexia nervosa = 1).

To develop a better understanding of the group  $\times$  methylation<sub>mean</sub> interaction, we extracted  $\beta$ -values using the MarsBaR toolbox (<http://marsbar.sourceforge.net/>) at a threshold of  $p < 0.001$  (uncorrected to account for greater anatomic variability) and subjected those to further testing using SPSS version 21.0 (SPSS Inc.). We examined associations with the physiologic and psychometric parameters body mass index standard deviation score (BMI-SDS),<sup>49</sup> EDI-2, BDI-II and SCL-90-R anxiety using Pearson  $r$  for each diagnostic group separately.

To probe the proposed serotonin-related epigenome-brain-behaviour pathway, we conducted a mediation analysis using the PROCESS package implemented in SPSS. To this end, we tested whether the link between alterations in *SLC6A4* methylation<sub>mean</sub> and elevated psychopathology (EDI-2 total, BDI-II and SCL-90-R anxiety) was mediated by altered rsFC in the salience network (indirect effect) in patients with anorexia nervosa using the bootstrapping procedure on 20000 samples to compute the lower and upper levels of the 95% confidence interval.

## Results

### Participants

As shown in Table 1, we found no significant group differences for age. As expected, patients with anorexia nervosa had lower BMI-SDS and a higher EDI-2 total, BDI-II and SCL-90-R anxiety scores than healthy controls. Patients with anorexia nervosa did not differ from healthy controls with respect to *SLC6A4* methylation<sub>mean</sub> or methylation<sub>CpG13</sub>. We did find that *SLC6A4* methylation<sub>mean</sub> was significantly associated with age ( $r_p = 0.22$ ;  $p = 0.021$ ), so we included age as a covariate in our main statistical models.

We investigated whether *SLC6A4* methylation<sub>CpG13</sub> was dependent on genetic variation, but based on publicly available data, we could not identify underlying methylation quantitative trait loci in blood (<http://mqtl.org>) or human brain tissue (<http://epigenetics.essex.ac.uk.mQTL>; Appendix 1, Supplemental Materials 2.6).

### Group comparison of independent components and association with methylation

A 2-sample  $t$  test of the 2 salience network components (IC13 and IC9) revealed increased rsFC in the left ( $t = 4.68$ ;  $p_{FWE} = 0.006$ ;  $k = 15$ ) and right ( $t = 4.88$ ;  $p_{FWE} = 0.014$ ;  $k = 7$ ) posterior insula and IC9 for patients with anorexia nervosa compared to healthy controls (step 1; Fig. 1). We identified no group difference for IC13. In step 2, we identified a group  $\times$  methylation<sub>mean</sub> interaction in the right dorsolateral prefrontal cortex (dlPFC) for IC9, controlling for age ( $t = 4.49$ ;  $p_{FWE} = 0.016$ ;  $k = 6$ ;  $x = 32$ ,  $y = 40$ ,  $z = 34$ ; Fig. 2, panel A). A detailed analysis of this interaction revealed that *SLC6A4* methylation<sub>mean</sub> was correlated positively with rsFC between this region and other areas of IC9 in patients with anorexia nervosa ( $r = 0.425$ ;  $p = 0.002$ ), but negatively in healthy controls ( $r = -0.408$ ;  $p = 0.002$ ; Fig. 2, panel B). In step 3, we

**Table 1: Demographic and clinical characteristics\***

Characteristic	Anorexia nervosa ( $n = 55$ )	Healthy controls ( $n = 55$ )	Statistics	
			$t$	$p$ value
<b>Demographic</b>				
Age, yr	16.15 $\pm$ 3.07	16.17 $\pm$ 3.07	-0.37	0.97
BMI-SDS	-3.21 $\pm$ 1.13	0.09 $\pm$ 1.04	-15.91	< 0.001
BMI	14.55 $\pm$ 1.35	20.68 $\pm$ 2.37	-16.44	< 0.001
<b>Clinical</b>				
Age at onset, yr	13.82 $\pm$ 2.63	NA		
EDI-2 total	202.27 $\pm$ 47.28	142.85 $\pm$ 26.82	-7.99	< 0.001
BDI-II	21.49 $\pm$ 10.92	5.78 $\pm$ 6.27	9.24	< 0.001
Anxiety (SCL-90-R)	0.81 $\pm$ 0.80	0.31 $\pm$ 0.41	4.04	< 0.001
<b><i>SLC6A4</i> DNA methylation</b>				
Mean <i>SLC6A4</i> methylation	5.45 $\pm$ 1.51	5.48 $\pm$ 2.28	-0.08	0.94
DNA methylation at CpG 13	8.05 $\pm$ 2.14	8.50 $\pm$ 7.11	-0.44	0.66

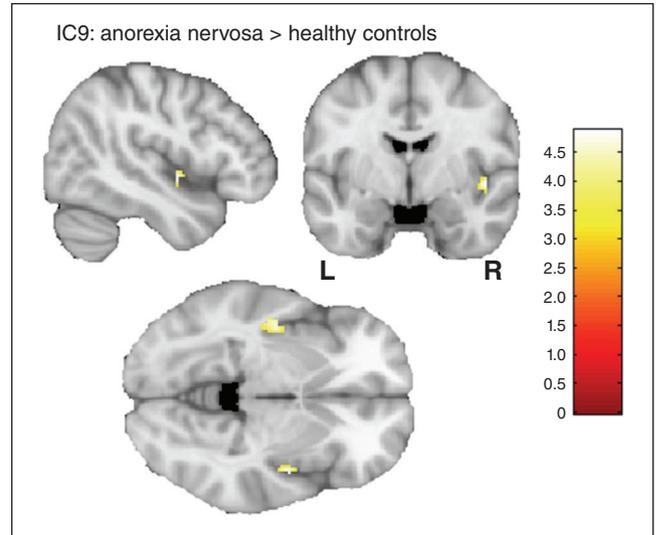
BDI-II = Beck Depression Inventory II; BMI = body mass index; BMI-SDS = body mass index standard deviation score; EDI-2 = Eating Disorder Inventory 2; NA = not applicable; SCL-90-R = Symptom Checklist 90 Revised.

\*Data are mean  $\pm$  standard deviation unless otherwise indicated.

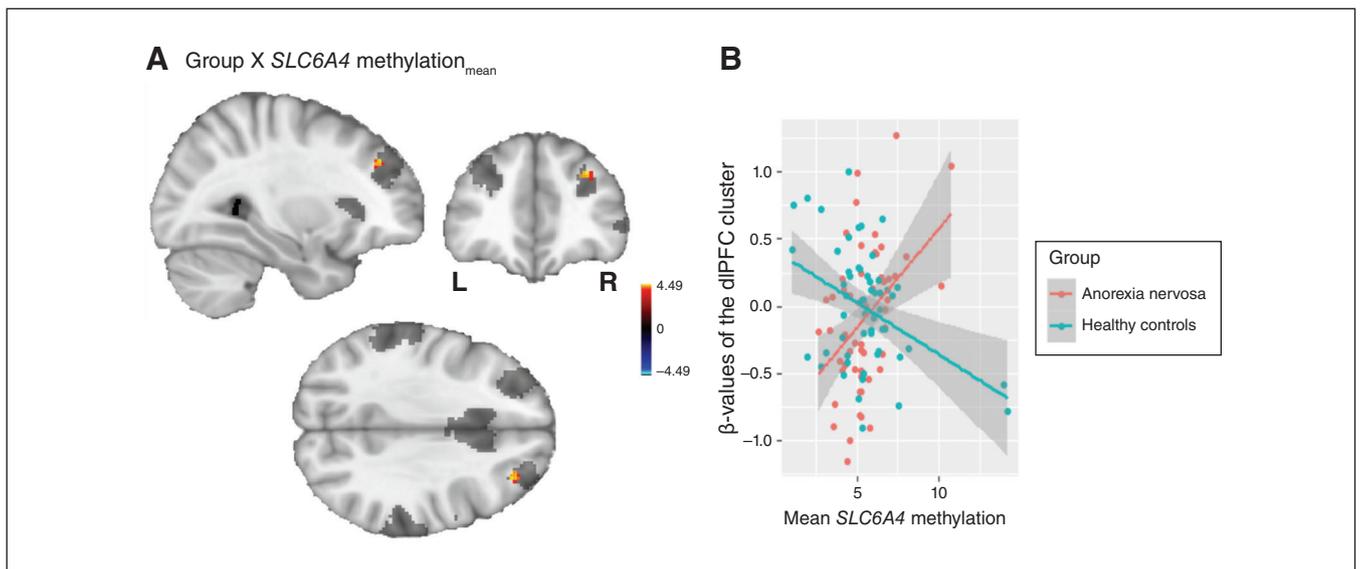
specifically tested the group  $\times$  methylation<sub>CpG13</sub> interaction and observed an identical neural pattern in the right dIPFC ( $t = 4.73$ ;  $p_{\text{FWE}} = 0.018$ ;  $k = 5$ ;  $x = 34$ ,  $y = 38$ ,  $z = 30$ ; Appendix 1, Supplemental Materials 2.1).

Control analysis in the visual network revealed no group  $\times$  methylation<sub>mean</sub> interaction, suggesting that the finding described above was specific to the salience network (step 4). Exclusion of current or previous smokers confirmed our initial results in the salience network ( $t = 5.09$ ;  $p_{\text{FWE}} = 0.007$ ;  $k = 13$ ;  $x = 30$ ,  $y = 40$ ,  $z = 32$ ). Exclusion of the participant who took SSRIs also had no influence on the results ( $t = 4.46$ ;  $p_{\text{FWE}} = 0.018$ ;  $k = 5$ ;  $x = 32$ ,  $y = 40$ ,  $z = 34$ ).

Increased rsFC between the significant cluster in the dIPFC (for which we reported the group  $\times$  methylation<sub>mean</sub> interaction) and other regions of the salience network was associated with eating disorder psychopathology, indicated by a correlation with the EDI-2 total score, but not with the BMI-SDS, BDI-II or SCL-90-R anxiety scores, in patients with anorexia nervosa ( $r_p = 0.349$ ;  $p = 0.010$ ; withstood Bonferroni correction for 4 tests). Mediation analysis showed that the effect of *SLC6A4* methylation<sub>mean</sub> on EDI-2 total (but not on BDI-II or on SCL-90-R anxiety) was mediated by alterations in the rsFC of the salience network in patients with anorexia nervosa (Table 2 and Appendix 1, Supplemental Materials 2.4).



**Fig. 1:** A 2-sample  $t$  test of the salience network component IC9 revealed increased functional connectivity between the left ( $t = 4.68$ ;  $p_{\text{FWE}} = 0.006$ ) and right ( $t = 4.88$ ;  $p_{\text{FWE}} = 0.014$ ) posterior insula and the rest of the component for patients with anorexia compared to healthy controls. FWE = family-wise error; IC9 = independent component 9.



**Fig. 2:** (A) Results of the group  $\times$  *SLC6A4* methylation<sub>mean</sub> interaction analysis in the salience network at the dorsolateral prefrontal cortex (dIPFC); for illustrative purposes shown at  $p < 0.001$ , uncorrected). The applied salience network mask<sup>45</sup> is depicted in transparent grey. The colour bar represents  $t$  values. (B) Plot of the association of the extracted  $\beta$ -values of the dIPFC cluster and mean *SLC6A4* methylation (in %) separately for patients with anorexia nervosa and healthy controls. methylation<sub>mean</sub> = mean methylation.

**Table 2: Mediation analysis between mean *SCL6A4* methylation score and outcome variables with rsFC of the salience network as the mediator**

Outcome	Total effect, 95% CI		Direct effect, 95% CI		Indirect effect, 95% CI	
	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
EDI-2 total	-2.642	14.385	-7.435	10.654	0.019	9.296
BDI-II	0.087	3.953	-0.561	3.706	-0.524	1.474

BDI-II = Beck Depression Inventory II; CI = confidence interval; EDI-2 = Eating Disorder Inventory 2; rsFC = resting-state functional connectivity.

## Discussion

The present study is, to our knowledge, the first to investigate a potential epigenome–brain–behaviour pathway in anorexia nervosa by examining the association between *SLC6A4* methylation and rsFC of the salience network in patients acutely ill with anorexia nervosa and healthy controls. At a group level, we observed increased rsFC in the salience network at the posterior insula in patients compared with controls, but (in agreement with previous reports<sup>19,50</sup>) no difference in *SLC6A4* methylation levels. However, we did identify a positive association between methylation levels and rsFC in the dlPFC and other regions of the salience network in patients with anorexia nervosa that was also associated with elevated eating disorder symptoms. In fact, alterations in salience network rsFC mediated the link between *SLC6A4* methylation changes and general eating disorder psychopathology, but not depressive or anxiety-related psychopathology. These results may suggest that this methylation-related rsFC pattern was specific to eating disorder symptoms. However, to confirm this notion, further research that includes other clinical groups is needed. In healthy controls, increased *SLC6A4* methylation levels were associated with reduced rsFC in this brain region. Importantly, these associations could be demonstrated for mean methylation as well as for CpG-specific methylation (*SLC6A4* methylation<sub>CpG13</sub>) at a locus with previous evidence for the correspondence of methylation levels across blood and brain tissue.<sup>16</sup> We detected no association between methylation and rsFC in a negative control network chosen a priori (the visual network), indicating that the association we found may have been specific for the salience network.

Alterations in the salience network have been described to underlie abnormal sensitivity to salient external or internal events, with severe consequences for cognition and behaviour across several mental disorders,<sup>51</sup> including anorexia nervosa.<sup>27</sup> The posterior insula, the location of the general group difference in the salience network, has been associated with pain and sensorimotor representation and is thought to function as a major interoceptive region.<sup>52</sup> In line with this, we have demonstrated altered functional network characteristics in the posterior insula in anorexia nervosa,<sup>53,54</sup> which may reflect inefficient information transfer to and from this brain region. Functional alterations in the insula have also repeatedly been shown in anorexia nervosa using task-based fMRI (for an overview of selected studies, see Appendix 1, Supplemental Materials 3). These dysfunctions in the insular cortices relate to impaired integration of visuospatial, homeostatic and interoceptive signals, which may account for some of symptoms in anorexia nervosa, such as body image distortions, elevated pain threshold and severe self-starvation.<sup>55</sup>

Our finding of methylation-related alterations in rsFC of the salience network, which were also associated with eating disorder severity in anorexia nervosa, fit well with a previously reported notion of a clinically relevant brain-mediated association between *SLC6A4* methylation and illness-related behaviour.<sup>29,31,56,57</sup> Previous neuroimaging studies in health and disease (for a review, see Palma-Gudiel and Fañanás<sup>18</sup>) — as well as our present findings in anorexia nervosa — suggest that increased *SLC6A4* methylation may be linked to heightened

activity and connectivity in brain regions related to the salience network in people with severe mental health conditions. In contrast to our observations for anorexia nervosa, we found a negative (rather than a positive) association between *SLC6A4* methylation and rsFC in the salience network in healthy controls. Similarly, Frodl and colleagues<sup>30</sup> demonstrated a negative association between methylation and neural reactivity in the posterior insula, pons and temporal pole when shifting attention away from emotional stimuli in a sample that included healthy controls and patients with major depressive disorder. The direction and exact localization of the association between *SLC6A4* methylation and brain functioning is still somewhat unclear and difficult to compare because of different fMRI methods used (resting state [seed-based<sup>31</sup> versus ICA] or task fMRI) and the contrast conditions in task fMRI.

Interestingly, although previous studies investigating *SLC6A4* methylation-related brain regions focused primarily on ventral-limbic parts of the brain such as the amygdala, we found a methylation-related increase in rsFC between the dlPFC and other regions of the salience network in anorexia nervosa. To further probe the specificity of this finding regarding the salience network, we also tested the frontoparietal network (network with the dlPFC as a central hub) as part of an additional follow-up analysis (Appendix 1, Supplemental Materials 2.3). Similar to the visual network (negative control network), we could not detect a significant association between methylation and rsFC in the frontoparietal network. Given that the methylation-related increase of rsFC was present in a section of the dlPFC belonging to the salience network but not for sub-regions of the dlPFC, which are part of the frontoparietal network, we suggest that the associations with *SLC6A4* methylation may be specific to the frontolimbic circuit. This circuit is a component of the salience network and part of the serotonin pathway (i.e., it is strongly innervated by serotonergic neurons and rich in various serotonin receptors).<sup>58</sup> With regard to processing emotions and saliency — a major function of the salience network — the dlPFC (and its coupling with limbic regions) is also engaged in bottom-up appraisal of emotions<sup>59</sup> and in emotion regulation via cognitive strategies.<sup>60</sup> Modulation of fronto-limbic circuits via the serotonergic system has previously been shown by studies focusing on the serotonin-transporter-linked polymorphic region. Based on this, a speculative interpretation of our findings could be that methylation-related increased involvement of the dlPFC in the salience network is related to elevated cognitive or self-control — a much debated characteristic of anorexia nervosa.<sup>2</sup> This tentative interpretation of the findings dovetails with the notion of anorexia nervosa as a model-disorder of increased cognitive control, an interpretation that may be particularly reflected in the tendency of patients to respond in a strategic rather than hedonic manner to salient stimuli.<sup>61,62</sup> Interestingly, in a recent study we discovered a sustained lateral prefrontal cortex responsivity in patients with anorexia nervosa when food-related social but also neutral stimuli were presented.<sup>62</sup> We speculated that this may reflect an inability of patients with anorexia nervosa to disengage cognitive control. In light of this, the current findings of increased rsFC between dlPFC and the rest of the salience network associated with *SLC6A4* — although correlational in nature — may be

interpreted as a tendency of anorexia nervosa patients to “stay in control,” which could be a neural downstream effect of elevated *SLC6A4* methylation, leading to unwanted effects such as increased eating disorder symptoms.

### Limitations

Interpretation of the present study rests on the following limitations. First, the cross-sectional nature of the study did not allow us to determine a causal link between epigenetic variation, brain network characteristics and the emergence of eating disorder symptoms, or whether the observed alterations were an effect of undernutrition. To shed light on temporal relationships, longitudinal studies are needed. Second, we assessed methylation measures peripherally, so our findings cannot be fully generalized to neural tissue. However, previous work could demonstrate a substantial association between methylation levels in blood and brain for *SLC6A4* methylation<sub>CpG137</sub><sup>16,17</sup> for which we could confirm our initial findings. Furthermore, although methylation at this site was somewhat low, which could add noise due to technical variation, the methylation levels in our study were in line with those of previous reports.<sup>63</sup> Third, the present study focused only on epigenetic variation and failed to incorporate genetic variability and other influencing factors such as stress and childhood adversity. Although the contribution of both epigenetic and genetic factors might be relevant to predicting the expression of *SLC6A4*,<sup>18</sup> *SLC6A4* methylation<sub>CpG13</sub> does not seem to be under strong genetic control. Fourth, rates of comorbidity were lower than previously reported,<sup>64</sup> which could have been due to the fact that no structured interview other than the SIAB-EX was used (Appendix 1, Supplemental Materials 1.1).

### Conclusion

If replicated, our findings suggest that a serotonin-related epigenome–brain–behaviour pathway is also relevant for anorexia nervosa. They shed light on the neurobiological process of how epigenetic variation of the *SLC6A4* gene may be associated with rsFC in the salience network that is specifically linked to eating-disorder symptoms. Such knowledge may spur future research into the potential of epigenetic markers to assess risk for disordered eating and pave the way for potential novel pharmacological and behavioural treatment strategies that modulate the methylation levels of specific genes.<sup>65,66</sup>

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**Affiliations:** From the Division of Psychological and Social Medicine and Developmental Neurosciences, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany (Boehm, Walton, Batury, Seidel, Geisler, King, Ehrlich); the Department of Psychology, University of Bath, Bath, UK (Walton); the Department of Psychology, Faculty of Human Sciences, Medical School Hamburg, Hamburg, Germany (Alexander); the Department of Psychotherapy and Psychosomatic Medicine, Faculty of Medicine, Technische Universität Dresden, Dresden, Germany (Weidner); the Department of

Child and Adolescent Psychiatry, Faculty of Medicine, Technische Universität Dresden, Dresden, Germany (Roessner); and the Eating Disorders Research and Treatment Center at the Department of Child and Adolescent Psychiatry, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany (Ehrlich).

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