Appendix 1 to Frodl T, Szyf M, Carballedo A, et al. DNA methylation of the serotonin transporter gene (*SLC6A4*) is associated with brain function involved in the processing of emotional stimuli.

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Supplementary methods

Emotional attention shifting task

In the emotional awareness and shifting attention task used in the *f*MRI experiment participants were asked to process visual stimuli.[23] The task was event-related and consisted of 180 pseudorandomized trials belonging to 2 groups. Each trial in the task lasted 4 s and consisted of a viewing stage where participants looked at a picture, and a response stage where they answered a question concerning the picture. The questions used in the task referred either to the emotional valence of a picture (Was it positive? Was it negative? Was it neutral?) or to its shape (Was it horizontal? Was it vertical?). Participants could only answer "yes" or "no" to all the questions depending on whether, in their opinion, the question stated truth or falsehood (Fig. S1). They answered by pressing 1 of 2 buttons on a 2-button response box from Current Design Inc. with their right hand.

Participants did not know before the start of each trial which of the 5 questions mentioned would be asked. To answer correctly they had to process information about the emotional valence and the shape of the picture until the question was asked. Then the whole attention would need to be focused on either the emotional content or the shape. Therefore, 2 groups of trials emerged in the task: those with shifting the attention to the picture's shape and those where the emotional information was processed (Fig. S1). Standardized training with training tasks outside the scanner preceded the fMRI.

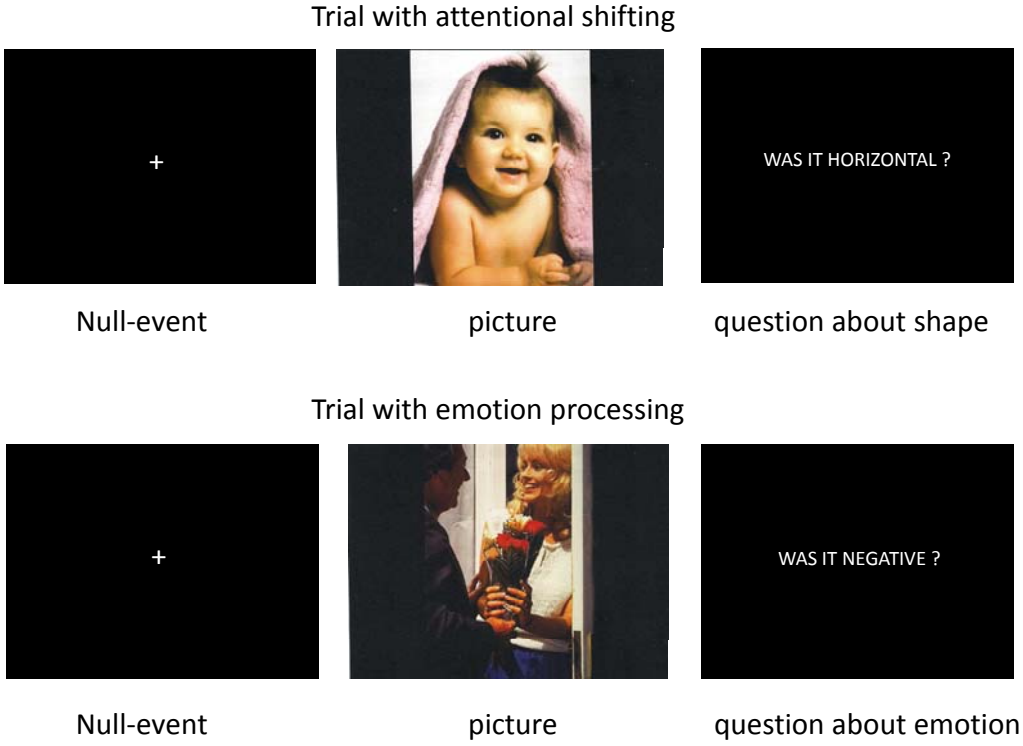

Fig. S1: Example of MRI trial. After seeing a cross as null-event, participants saw an image with either positive, neutral, or negative emotional valence that could be presented either horizontally or vertically. After that they were asked to either focus on the emotional content or the geometrical presentation of the image.

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Pictures used in the experiment were taken from the well-validated International Affective Picture System (IAPS) database and were positive, negative or neutral in emotional valence as well as horizontal or vertical in shape, and there were 60 unrepeated pictures in each valence category. The valence of IAPS pictures is described on a scale from 1 to 9, where 1 represents very negative and 9 very positive. Pictures in the interval from 1 to 3 were classified as negative, from 4 to 6 as neutral and from 7 to 9 as positive. The pictures selected for the experiment were as close as possible to 1, 9 and 5 for the negative, positive and neutral categories, respectively. To ensure that the chosen pictures would have a consistent appraisal in healthy population, the ones with minimal standard deviation in emotional valence and the ones judged similarly by men and women were selected. Since the examined group consisted of emotionally vulnerable participants, negative pictures presenting highly disturbing content were omitted after a consultation with a psychiatrist. In the end, the respective mean valence values for the negative, positive and neutral category were 2.54 ± 0.34, 7.64 ± 0.34 and 4.97 ± 0.23, respectively. The selected pictures of different valence and shape were randomly and equally distributed across the 2 types of trials. The trials were intervened with jittered null events, which consisted of a white cross on black background presented for 2 s on average.

Preprocessing

Preprocessing steps for fMRI data included realignment to correct for motion. Participants were excluded when movement parameters exceeded 1 slice thickness (4.8 mm). Three patients and 1 control had movement artifacts during the scanning, so in total fMRI data from 25 patients and 35 healthy controls were analyzed. Then co-registration of each participant's structural image to the mean of the motion-corrected functional images, slice time correction, spatial normalization and smoothing using an 8 mm full-width at half-maximum (FWHM) Gaussian kernel were applied. Data were analyzed using Statistical Parametric Mapping (SPM8). Motion correction values were added as a covariate. In first-level analyses *t* test contrasts were calculated contrasting positive or negative picture stimuli versus neutral picture stimuli, shifting attention away from negative stimuli versus shifting attention away from positive stimuli, and judging the emotional content versus judging the geometry of the images for each emotional valence separately. In consequence, a set of 6 subsequent contrasts was acquired for each individual.

DNA methylation

We previously targeted the entire 214-625 bp regulatory region upstream of the *SLC6A4* gene promoter (CpG 1–24).[11] DNA methylation of this region resulted in loss of promoter activity in transient transfection promoter-luciferase reporter assays.[11] In this study we used whole blood DNA, hypothesizing that differential DNA methylation of these sites would be detectable in whole blood DNA as it was in selected white blood cell subtypes. We targeted CpG sites 5–15, as CpG sites within this region were previously most strongly associated with in vivo measures of brain serotonin synthesis, in particular in CpG sites 5, 6, 11 and 12,[11] and thus most relevant to test our current hypotheses. The DNA methylation pattern in the target region of the *SLC6A4* gene promoter was investigated using the following 3 sets of outside primers and 4 sets of nested primers: Out F1&2 5′-TGTAGTTGGTTAATAAAATGAGAATTAGTT-3′, Out R1&2 5′-AAATCCTAACTTTCCTACTCTTTAAC-TTTA-3′, Out F3 5′-TTTTAGGAAGAAAGAGAG-AGTAGTTTT-3′, Out R3 5′-CCAAAAAACTCTTAAAAAA-TTTTTAC-3′, Out F4 5′-TTTGT-TTTTTTGTGTAGTTTTTTTT-3′, Out R4 5′-CTCACATAATCTAATCTC-TAAATAACC-3′, Nest F1 5′-TTTTTTATTGTGGAAGTTTTTATTGTG-3′, Nest R1 5′-CTCTCTCTTTCTTCCT-AAAACCTAACA-3′, Nest F2 5′-TTGTTAGGTTTTAGGAAGAAAGAGAGA-3', Nest R2 5′-AAAAAAA-ACTACACAAAAAAAACAAATATAC-3′, Nest F3 5′-TTTTAGGAAGAAAGAG-AGAGTAGTTTT-3′, Nest R3 5′-AAATCCTAACTTTCCTACTCTTTAACTTTA-3′, Nest F4 5′-TAAAGTTAAAGAGTAGGAAAGTTAGGATTT-3′, and Nest R4 5′-ACCCCAAAACCA-AAAAAAAA-3'. The nested reverse primers were biotinylated for pyrosequencing. DNA was treated with sodium bisulfite, and 2 rounds of polymerase chain reaction (PCR) amplification were performed, as previously described. We used 15 µL of the PCR products to perform pyrosequencing using PyroMarkQ24 (Qiagen) according to the manufacturer's protocol.

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Table S1: Behavioral data during the fMRI experiment*

Behaviour	Group, mean ± SD		Statistic	*p* value
	Patients	Controls		
Incorrect judgment				
Positive images as neutral	4.8 ± 2.3	2.2 ± 2.2	$F_{1,54}$ = 15.1	< 0.001
Neutral images as not neutral	2.7 ± 2.4	4.7 ± 3.5	$F_{1,54}$ = 8.5	0.005
Negative images as neutral	1.8 ± 2.2	1.1 ± 1.3	$F_{1,54}$ = 0.17	0.20
Positive images as negative	0.65 ± 1.3	0.65 ± 1.0	$F_{1,54}$ = 0.54	0.57
Neutral images as negative	1.1 ± 1.2	0.94 ± 1.3	$F_{1,54}$ = 0.09	0.76
Neutral images as positive	2.8 ± 2.3	3.0 ± 2.2	$F_{1,54}$ = 0.63	0.43
Negative images as positive	0.46 ± 1.0	0.41 ± 1.3	$F_{1,54}$ = 0.30	0.59
Reaction time				
Shifting attention to geometrical figure after neutral pictures	1031.6 ± 281.6	764.9 ± 226.0	$F_{1,54}$ = 12.0	0.001
Shifting attention to geometrical figure after positive pictures	1033.1 ± 285.4	776.1 ± 235.4	$F_{1,54}$ = 9.4	0.003
Shifting attention to geometrical figure after negative pictures	1132.4 ± 266.2	826.9 ± 273.9	$F_{1,54}$ = 12.3	0.001
Focusing on emotion after neutral pictures	1159.3 ± 255.4	968.9 ± 232.1	$F_{1,54}$ = 5.2	0.027
Focusing on emotion after positive pictures	883.4 ± 213.1	700.5 ± 230.0	$F_{1,54}$ = 7.7	0.008
Focusing on emotion after negative pictures	1022.0 ± 234.1	830.7 ± 244.1	$F_{1,54}$ = 5.9	0.019

SD = standard deviation.
*Patients with major depressive disorder showed a processing bias toward judging stimuli as being more negative and took longer in particular to shift attention toward the geometrical shape of the image than healthy controls.

Table S2: Clusters surviving a $p < 0.001$ uncorrected threshold to allow comparison with the broader literature (part 1 of 4)

Contrast, region	MNI coordinates			k	*t*	*p* value†
	x	*y*	*z*			
Valence interaction, none						
Emotion – neutral pictures						
Right postcentral*	24	–25	55	394	4.74	< 0.001
Left precuneus extending to middle cortex cinguli*	–15	–37	58	—	4.42	—
Right motor area*	9	–22	55	—	4.36	—
Right hippocampus*	21	–37	16	85	4.22	0.045
Left superior temporal lobe	–60	–40	16	18	4.11	—
Right dorsomedial frontal lobe	6	56	31	19	4.0	—
Left precuneus	–27	–49	19	14	3.95	—
Left middle temporal lobe	–45	–58	13	31	3.86	—
Left insula	–42	–16	1	19	3.85	—
Left cerebellum	–15	–31	–26	21	3.84	—
Right superior frontal cortex	15	–13	61	51	3.83	—
Left precuneus	–6	–52	37	28	3.77	—
Main MDD < control						
Cerebellum	0	–55	–8	91	4.13	0.036
Negative – neutral pictures						
Diagnosis x methylation interaction						
Hippocampus*	15	–7	–8	266	4.24	< 0.001
	–6	–13	–14			
Right angular	51	–58	34	11	4.12	ns
Right lingual, right parahippocampal	12	–34	–17	23	3.49	ns
High > low methylation						
Left insula*	–27	8	16	160	4.54	0.002
Left inferior frontal operculum*	–45	2	17	—	4.18	—
	–39	17	22		4.09	
Right rolandic operculum	42	–1	22	21	4.18	0.53
Vermis	0	–55	–5	30	3.55	0.34

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Table S2: Clusters surviving a $p < 0.001$ uncorrected threshold to allow comparison with the broader literature (part 2 of 4)

Contrast, region	MNI coordinates			k	*t*	*p* value†
	x	*y*	*z*			
Low > high methylation, none						
MDD > control						
Left paracentral lobule	–12	–19	64	24	4.37	ns
Right SMA	12	5	61	11	3.85	ns
Left cerebellum	–12	–31	–32	19	3.84	ns
Left superior frontal lobe	–24	–4	55	10	3.72	ns
Control > MDD, none						
Low > high methylation in controls, none	9	2	52	18	3.86	ns
High > low methylation in controls						
Left frontal inferior operculum*	–45	5	16	296	4.79	< 0.001
Left fusiform gyrus*	–24	–37	–17	630	4.68	< 0.001
Left rectus	–12	41	–17	20	3.84	ns
Right rolandic operculum	48	–4	19	13	3.78	ns
Right frontal medial orb	6	47	–14	13	3.62	ns
High methylation: control > MDD, none						
High methylation: control < MDD, none						
Low methylation: control > MDD						
Vermis	3	–52	–2	19	3.78	ns
Low methylation: control < MDD						
Left cerebellum/ hippocampus*	–9	–34	–20	648	4.96	< 0.001
Left middle temporal lobe*	–54	–61	16	120	4.92	< 0.001
Left superior temporal lobe	–63	–40	16	18	3.96	ns
Left superior frontal lobe	–21	–1	58	16	3.81	ns
Positive – neutral pictures						
Diagnosis x methylation interaction						
Right hippocampus*	15	–7	–11	52	4.47	0.06‡
High > low methylation						
Right inferior occipital lobe	27	–91	–5	21	3.84	ns
High < low methylation						
Right frontal inferior trigonum	51	32	4	14	3.84	ns
MDD > control						
Right heschl gyrus, right insula	30	–37	19	37	3.65	ns
MDD < control, none						
High > low methylation in controls						
Right hippocampus*	15	–10	–14	117	5.24	0.012
Left amygdala/hippocampus*	–21	–1	–11	111	4.49	0.015
High > low methylation in MDD						
Right cerebellum	12	–79	–17	28	3.77	ns
Right lingual cortex	24	–94	–8	25	3.76	ns
High < low methylation in controls						
Right paracentral lobule	6	–28	58	13	3.64	ns
High < low methylation in MDD						
Right inferior frontal trigonum	48	35	1	26	3.81	ns
High methylation: control > MDD						
Right hippocampus	12	–10	–14	14	3.68	ns
High methylation: control < MDD						
Right paracentral lobule	3	–37	58	31	3.75	ns
Low methylation: control > MDD, none						
Low methylation: control < MDD						
Right hippocampus	21	–37	19	101	4.13	0.022
Left superior temporal lobe	–60	–40	19	10	4.22	ns
	–42	–13	–2	18	3.72	ns
Right superior temporal lobe	54	–10	–8	15	3.69	ns
Left DMPFC	–12	38	22	11	3.62	ns
	–6	44	22	13	3.56	ns

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Table S2: Clusters surviving a $p < 0.001$ uncorrected threshold to allow comparison with the broader literature (part 3 of 4)

	MNI coordinates					
Contrast, region	*x*	*y*	*z*	k	*t*	*p* value†
Focusing on geometrics following negative pictures – focusing on geometrics following positive pictures						
Interaction, none						
High < low methylation						
Right rolandic operculum/insula*	39	–19	16	50	5.71	0.005
Right fusiform gyrus	30	–55	–5	21	4.30	ns
Right DMPFC	12	59	7	14	3.53	ns
Control > MDD						
Right hippocampus	9	–16	–20	27	4.41	ns
Control < MDD, none						
High < low methylation in MDD						
Right inferior orbitofrontal cortex/insula	48	23	–8	50	4.44	ns
High > low methylation in MDD						
Right pons extending to hippocampus/parahippocampus*	9	–19	–23	123	4.5	0.019
Left fusiform gyrus	–39	–43	–26	38	4.4	ns
Right cerebellum	21	–46	–29	17	3.69	ns
Focusing on geometrics following positive pictures – focusing on geometrics following neutral pictures						
Interaction, none						
MDD < control						
Left inferior frontal operculum	–48	8	10	24	3.98	ns
MDD > control						
Right middle temporal cortex	51	–7	10	11	3.83	ns
High < low methylation						
Right lingual cortex	27	–91	–8	31	4.00	ns
High > low methylation, none						
Focusing on geometrics following negative pictures – focusing on geometrics following neutral pictures						
Interaction						
Left thalamus	0	–4	–8	30	3.77	ns
MDD < control, none						
MDD > control						
Left inferior temporal lobe						ns
High < low methylation, none						
High > low methylation, none						
Focusing on geometrics – focusing on emotional content both following neutral pictures						
Interaction, none						
High < low methylation						
Right superior temporal lobe*	54	–34	16	92	5.23	0.029
Left insula	–36	–10	19	50	4.90	ns
Right postcentral	57	–13	19	13	3.96	ns
Right middle temporal lobe	69	–25	–11	11	3.76	ns
High > low methylation, none						
MDD > control, none						
MDD < control						
Right fusiform gyrus	42	–37	–23	28	4.1	ns
High < low methylation in patients						
Left insula*	–36	–7	19	51	5.00	0.05
Right middle temporal lobe*	48	–46	10	78	4.81	0.05
	69	–25	–14	11	4.21	ns
High < low methylation in controls						
Right middle cortex cinguli	9	38	31	23	3.87	ns
Left middle frontal cortex	–33	17	31	12	3.6	ns
High > low menthylation in MDD, none						
High > low methylation in controls, none						
Focusing on geometrics – focusing on emotional content both following negative pictures						
Interaction, none						
High > low methylation, none						
High < low methylation, none						

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Contrast, region	MNI coordinates			k	*t*	*p* value†
	x	*y*	*z*			
Control > MDD, none						
Control < MDD, none						
Focusing on geometrics – focusing on emotional content both following positive pictures						
Interaction, none						
High > low methylation, none						
High < low methylation						
Pons, brainstem, left cerebellum, parahippocampus*	–12	–28	–26	172	4.51	0.004
Control > MDD						
Vermis	6	–52	4	15	3.57	ns
Control < MDD, none						
High > low methylation in controls, none						
High < low methylation in controls, none						
High > low methylation in MDD						
Right inferior orbitofrontal cortex	45	23	–11	40	3.83	ns
High < low methylation in MDD						
Pons, brainstem, left cerebellum, parahippocampus*	–15	–25	–26	128	4.47	0.013
Right middle frontal cortex	33	2	55	10	3.72	ns

DMPFC = dorsomedial prefrontal cortex; FDR = false discovery rate; FWE = family-wise error; MDD = major depressive disorder; MNI = Montreal Neurological Institute; ns = nonsignificant; SMA = supplementary motor area.
*Survived FWE correction.
†FWE-corrected.
‡FDR-corrected.