Differential DNA Methylation Is Associated With Hippocampal Abnormalities in Pediatric Posttraumatic Stress Disorder

Supplementary Information

Participants

Exclusion criteria for youth with PTSD in both cohorts included imminent suicidality, history of psychotic disorder, substance abuse or dependence; IQ<70; unstable medical condition; recent use of psychotropic medication (past 4 weeks; 6 weeks for fluoxetine); MRI contraindication; and possibility of pregnancy in females. NTC and TC were screened for trauma history with use of the Children's Revised Impact of Event Scale (CRIES) and if traumatized, screened on PTSD symptoms (1, 2). NTC an TC participants were free of any history of mental illness. In both cohorts, youth with PTSD participated in ongoing studies aiming to investigate biological changes in relation to trauma exposure and PTSD. Clinical characteristics of both cohorts are shown in Table 1A and Table 1B. In the Dutch cohort, not all youth participated in the MRI analysis (N=52). In the USA cohort, all 44 youth, including NTC youth, participated in the MWAS and MRI portions of the study. In both cohorts written parental consent and youth assent were obtained for all participants.

Clinical and Behavioral Assessments

In both cohorts, participants and their caregivers underwent a traumatic events and psychiatric screen by trained child and adolescent psychiatrists or psychologists. The Clinician-Administered PTSD Scale for Children and Adolescents (CAPS-CA) was used to determine PTSD diagnosis based on DSM-IV-TR criteria(3, 4). A PTSD diagnosis required at least five symptoms, including at least one from each symptom category. In the Dutch cohort, in addition to the CAPS-CA, interview caregiver information based on the PTSD section of the Anxiety disorders interview schedule for DSM-IV (ADIS) was used (5). In the USA cohort the CAPS-CA was not obtained for the first four PTSD participants included in the analysis. PTSD severity was additionally examined using the UCLA PTSD Reaction Index (PTSD-RI) (6). For the PTSD-RI, the greater score between the youth and caregiver report for each item was used as this was most strongly correlated with CAPS scores. In both cohorts, information about youth depressive and anxiety symptoms were measured using youth and caregiver reports. Both cohorts used different measures to assess these symptoms. In the Dutch cohort, information about internalizing and externalizing symptoms were obtained with the Revised Children's Anxiety and Depression Scale (RCADS), the Child Behavioral Checklist (CBCL) and Youth Self Report (YSR) (7-11). In the American cohort, the Kiddie Schedule for Affective Disorders and Schizophrenia (KSADS) (12) was used for

general psychiatric screening. Information about internalizing symptoms were obtained with use of the Mood and Feelings Questionnaire (MFQ) (13) and Screen for Child Anxiety Related Emotional Disorders (SCARED) (14).

DNA Acquisition and Extraction

Genomic DNA samples were resolved on a 1% agarose gel to verify that the DNA was of high molecular weight and quantified using Qubit (Qiagen, USA).

Bisulfite Conversion and Generation of Methylation Signal

In both cohorts, five hundred nanograms of genomic DNA was sodium bisulfite–treated for unmethylated cytosine (C) to thymine (T) conversion using the EZ DNA Methylation-Gold kit (Zymo Research). Briefly, converted DNA was amplified, fragmented, and hybridized. The converted DNA was then scanned using the HumanMethylation EPIC/850 BeadChip (Dutch cohort) and 450 BeadChip (USA cohort) following the manufacturer's guidelines. Illumina recently replaced the HumanMethylation450 BeadChip (450K) with the EPIC BeadChip, which nearly doubles the measured CpG sites to >850,000. However data obtained from two platforms is comparable within cohorts (15).

Quality Control and Data Processing

Quality control of the raw data was performed to determine the success of the bisulfite conversion and subsequent array hybridization using Methylaid package (v.1.16.0) (16). Four samples from the Dutch cohort and one sample from the American cohort were removed before further analysis during this step due to poor sample quality. The removed samples were part of the PTSD group, and were excluded for further analysis. See table 1, for the resulting cohort sizes. Next, both the Dutch and American data sets were normalized using quantile
normalization implemented in the Minfi package (v.1.22.1) (17). Furthermore, based on Illumina's recommendations, probes present on the X or Y chromosomes, probes represented by a SNP, and probes known to be susceptible for cross hybridization were removed. Density plots were made to evaluate whether the normalization procedure was effective. In order to explore our datasets further, we applied a principal component analysis (PCA) on the raw and normalized datasets and we evaluated the first four components in relation to potential technical or biological confounders.

Image Preprocessing and Voxel-Based Morphometry

Preprocessing and VBM were performed using the VBM8 toolbox (http://dbm.neuro.uni-jena.de/vbm/) in Statistical Parametric Mapping (Wellcome Department of Imaging Neuroscience, London, UK), which

was executed in Matlab (Mathworks, Sherborn, MA). Standard VBM8 toolbox parameters were used for preprocessing. Images were bias corrected, tissue classified, and normalized to MNI space using linear (12-parameter affine) and nonlinear transformations including high-dimensional DARTEL within a unified model. Standard values were selected to bias regularization (0.0001) and FWHM cutoff (60 mm). Denoising was performed with optimized Rician non-local means and HMRF weighting of 0.15. Output normalized GMVs were modulated for nonlinear (Jacobian) components only, resulting in images corrected for total brain volume and smoothed with a 6-mm FWHM Gaussian filter. Final voxel resolution was 1.5 × 1.5 × 1.5 mm.

Demographic and mental health measures of participating youth

Results are shown in Table 1. In the Dutch cohort (n= 224), 33 % of the youth were diagnosed with PTSD, 33.5% consisted of traumatized youth without PTSD (TC) and 33.5% consisted of healthy non traumatized comparison (NTC) youth. Compared to the PTSD youth, TC and NTC were more likely to have a Caucasian ethnicity, and NTC were slightly younger of age (e.g. -1.3 years of difference with the PTSD youth). In the American cohort (n=42), 52.4% within the USA cohort were diagnosed with PTSD, the other 47.6% consisted of NTC. In this cohort there were no significant group differences in sex, ethnicity, handedness distribution, age, or IQ. In both groups, youth with PTSD also reported comorbid internalizing and externalizing mental health problems.

Supplementary Tables and Figures

Table S1: DMRs identified in identified in the PTSD vs non-traumatized control groups, and PTSD vs traumatized control groups in the Dutch cohort.

PTSD Youth vs	**Gene**	**Chr: start-end**	**Area**	**L**	**Cluster (L)**	***p*-value**	**FWER**	**Direction**
NTC Youth	*TNXB*	6: 32064573-32064660	0.08184208	7	63	1,68E-05**	0.03	PTSD > NTC
	PM20D1	1: 205818956-205819609	0.89112306	12	15	2,24E-05**	0.04	NTC > PTSD
	TNXB	6: 32063901-32064258	0.71518432	11	63	6,50E-05	0.10	PTSD > NTC
	SLC39A4	8: 145638434-145639652	0.49039230	7	14	7,73E-05	0.13	PTSD > NTC
	MRI1	19: 13875014-13875111	0.22034351	2	12	9,41E-05	0.15	NTC > PTSD
	HOOK2	19: 12876846-12877188	0.32988402	4	4	0.00016	0.24	PTSD > NTC
	KRTCAP3	2: 27665079-27665150	0.34323537	5	12	0.00016	0.24	PTSD > NTC
	DUSP22	6: 291687-293285	0.51044509	10	10	0.00020	0.33	PTSD > NTC
	KLRC4-KLRK1	12: 10563981-10564015	0.19874756	2	2	0.00024	0.32	PTSD > NTC
	SH2D1B	1: 162382662-162383000	0.25738391	3	6	0.00024	0.33	NTC > PTSD
PTSD Youth vs	*HOOK2*	19: 12876846-12877188	0.58247642	4	4	7.19E-07**	0.002	TC > PTSD
TC Youth	*SLC39A4*	8: 145638434-145639652	0.48956705	7	14	3.88E-05	0.104	PTSD > TC
	TNXB	6: 32063901-32064258	0.66185491	11	63	5.25E-05	0.13	PTSD > TC
	DUSP22	6: 291687-293285	0.60519676	10	10	6.61E-05	0.168	PTSD > TC
	TNXB	6: 32064573-32064660	0.39522854	7	63	0.00016103	0.352	PTSD > TC

	-	12: 7781004-7781431	0.30113510	5	6	0.00019985	0.398	PTSD > TC
	-	6: 31650760-31650930	0.38106164	7	21	0.00019482	0.406	PTSD > TC
	-	13: 50194322-50194643	0.24197756	3	3	0.00018763	0.414	PTSD > TC
	KLRC4-KLRK1	12: 10563981-10564015	0.18006498	2	2	0.00023220	0.45	PTSD > TC
TC Youth vs NTC Youth	PM20D1	1: 205818956-205819609	1.007570321	12	15	2.53E-06	0.016	TC > NTC
	MYOM2	8: 2075209-2075820	0.370255087	4	5	2.12E-05	0.128	NTC > TC
	MRI1	19: 13875014-13875111	0.217915630	2	12	2.65E-05	0.14	NTC > TC
	HOOK2	19: 12876846-12877188	0.323727987	4	4	9.58E-05	0.452	TC > NTC
	HCG4P6	6: 29894050-29894228	0.323727987	6	23	0.00012673	0.546	NTC > TC
	GNE	9: 36276879-36277313	0.304210036	6	8	0.00018204	0.652	NTC > TC
	ZNF718	4: 124232-124344	0.274002874	5	8	0.00018172	0.656	TC > NTC
	NINJ2	12: 739953-740338	0.274002874	5	5	0.00018425	0.66	TC > NTC
	STAP2	19: 4328745-4328818	0.200618804	3	4	0.00024999	0.768	TC > NTC
	-	2: 731215-732037	0.337826311	8	9	0.00022913	0.77	TC > NTC

Top 10 DMRs of association analyses of 1) PTSD vs. NTC youth, (2) PTSD vs TC youth and 3) TC vs NTC youth. Detected DMRs (L>1) using minfi's "bumphunter" function; chr: chromosome and position; area: area bump; L: number of probes in DMR; DMR:Differently methylated region ; cluster(L): number of probes in cluster; FWER = Family-Wise Error Rate; PTSD = Post-Traumatic Stress Disorder; NTC = Non-Traumatized Controls, TC = Traumatized controls. **indicates a significant result.

Table S2: Top 10 DMRs identified in the PTSD vs non-traumatized control groups, and PTSD vs traumatized control groups in the USA cohort.

PTSD Youth vs	Gene	Chr: start-end	Area	L	Cluster (L)	*p*-value	FWER	Direction
NTC Youth	*CYP2E1*	10: 135341528-135343280	0.985407559	11	11	2.14E-05	0.1	PTSD > NTC
	PM20D1	1: 205818956-205819492	0.653954047	7	10	6.58E-05	0.276	NTC > PTSD
	SH2D4B	10: 82295394-82296191	0.535736945	6	6	0.00010352	0.386	PTSD > NTC
	DUSP22	6: 291687-292823	0.682382028	9	10	0.00011905	0.434	NTC > PTSD
	GDF7	2: 20870087-20871401	0.683556474	9	9	0.00011905	0.434	NTC > PTSD
	KLHL35	11: 75139390-75139736	0.434895571	4	4	0.00018597	0.598	PTSD > NTC
	TNXB	6: 32064153-32064491	0.527903349	8	68	0.00033977	0.778	PTSD > NTC
	IGF2BP1	17: 47091339-47092272	0.462007265	7	9	0.00047841	0.854	NTC > PTSD
	NAP1L5	4: 89619038-89619053	0.284233465	3	21	0.00058859	0.908	PTSD > NTC
	TACSTD2	1: 59043070-59043280	0.377549355	6	15	0.00084813	0.94	NTC > PTSD

Top 10 DMRs of association analyses of PTSD vs. NTC youth; Chr = chromosome; DMR = Differently methylated region; FWER = Family-Wise Error Rate PTSD = Post-Traumatic Stress Disorder; NTC = Non-Traumatized Controls.

Table S3: Top 5 DMPs identified in the PTSD vs non-traumatized control groups, and PTSD vs traumatized controls group in the Dutch cohort

	Gene	Probe	Chr	Position	m-value	FDR	Log Beta	Delta Beta	Gene Feature
PTSD Youth vs. NTC Youth	*CRHBP*	cg26196496	5	76247679	2,63E-08	0,0204**	0,1314	0,1634	TSS1500
	LINC00379	cg22797297	13	91807638	7,60E-07	0,1785	-0,0828	-0,1111	Body
	-	cg11717701	4	55408833	9,02E-07	0,1785	0,0538	-0,0002	-
	RN5S96	cg17852114	2	69410331	9,19E-07	0,1785	-0,0135	-0,0130	Body
	AC092567.1	cg12139537	2	62972370	1,36E-06	0,1937	-0,0257	-0,0060	body
PTSD Youth vs. TC Youth	-	cg21972431	7	17812356	2,30E-08	0,0179**	-0,0275	-0,0108	-
	PPP1R16B	cg12221474	20	37433936	2,75E-07	0,0675	-0,0246	-0,0208	TSS1500
	-	cg0694353	6	142007733	3,88E-07	0,0675	-0,1512	-0,1417	-
	ZEB2	cg20171775	2	145228686	4,37E-07	0,0675	0,1088	0,0704	3'UTR
	FAM180A	cg11015893	7	135433540	4,60E-07	0,0675	0,0279	0,0049	1stExon
TC Youth vs. NTC Youth	*KIAA1949*	cg18335326	6	30653659	2,51E-06	0,7123	0,0290	0,0243	Body;1stExon
	URI1	cg23798674	19	30433017	3,11E-06	0,7123	-0,0100	-0,0089	TSS200
	CNN2	cg10658703	19	1033242	5,27E-06	0,7123	0,0209	-0,0208	Body
	RAD51B	cg13898955	14	68555990	7,19E-06	0,7123	-0,0453	-0,0293	Body
	-	cg20230271	13	95190074	8,01E-06	0,7123	0,0307	0,0358	-

Top 5 DMPs of association analyses of (1) PTSD vs NTC youth, (2) PTSD vs TC youth and 3) TC vs NTC youth. Gene: UCSC Reference Gene Name, chr: chromosome; m-value: adjusted p-value (Mval); DeltaBeta: delta differences between groups, based on average β-value. DMP: Differently methylated position: Gene feature: gene feature according Illumina manifest. PTSD = Post-Traumatic Stress Disorder; NTC = Non-Traumatized Controls, TC = Traumatized controls. **indicates a significant result.

Table S4: Top 5 DMPs identified in the PTSD vs non-traumatized control groups in the USA cohort

PTSD Youth vs. NTC Youth	Gene	Probe	Chr	Position	m-value	FDR	Log Beta	Delta Beta	Gene Feature
	SORBS1	cg12639763	10	97321104	4.94E-07	0.327068412	-0.0073432	-0.0061897	5' UTR
	CACNG8	cg17780246	19	54481467	2.69E-06	0.254915768	-0.0677602	-0.061728	Body
	LPGAT1	cg05959111	1	211999985	2.97E-06	0.254915768	-0.0585573	-0.0588481	Body
	DLGAP2	cg05455971	8	1616422	2.51E-06	0.254915768	0.04179431	0.03864045	Body
	SHPK	cg02501127	17	3530781	5.34E-06	0.105837742	-0.0266202	-0.0254701	Body

Top 5 DMPs of association analyses of PTSD vs. NTC youth; Gene: UCSC Reference Gene Name, Chr = chromosome; Delta Beta = delta differences between groups, based on average β-value. DMP: Differently methylated position Gene feature: gene feature according Illumina manifest. PTSD = Post-Traumatic Stress Disorder; NTC = Non-Traumatized Controls.

Table S5: Demographic Information, Trauma History and Clinical Characteristics of the subset of youth that participated in the Post Hoc analysis in the Dutch cohort

	PTSD Youth (n=45)
Sex	
Boys	17 (37.78%)
Girls	28 (62.22%)
Age	12.60 (2.95)
Ethnicity	
Caucasian	22 (48.49%)
Other	23 (51.11%)
Left Handed	4 (8.89%)
Index Trauma	
Interpersonal Violence	26 (57.78%)
Sexual Abuse	9 (20%)
Severe Accident/medical trauma	1 (2.22%)
Other (Traumatic News, Natural Disaster)	7 (15.56%)
Comorbid Diagnoses	
Internalizing	12 (26.67%)
Externalizing	9 (20%)
CAPS-CA Severity Score	53.58 (24.19)

Continuous variables presented as mean (standard deviation); categorical variables presented as frequency (percentage).

Table S6: Lambda's in the Dutch cohort reflecting the inflation index.

	Dutch cohort	**USA cohort**
PTSD Youth vs. NTC Youth	1,043246	1.00317
PTSD Youth vs. TC Youth	1,033907	
TC Youth vs. NTC Youth	1,041038	

The lambda's of the qq-plots after using the BACON, indicated absence of type-I error inflation

Figure S1: Results for the comparison between PTSD cases vs HC in the Dutch cohort. CpGs in or near CRHBP. Above providing log10(p values) with individual CpGs indicated by dots, color coded based on pairwise correlation with neighboring CpGs. The second part (ENSEMBL genes, CG Island, BroadChromHMM) presents the annotation tracks for the plotted genomic region. The final part of the figure (SNP USCS, and below) presents the pairwise correlation matrix across the displayed CpGs.

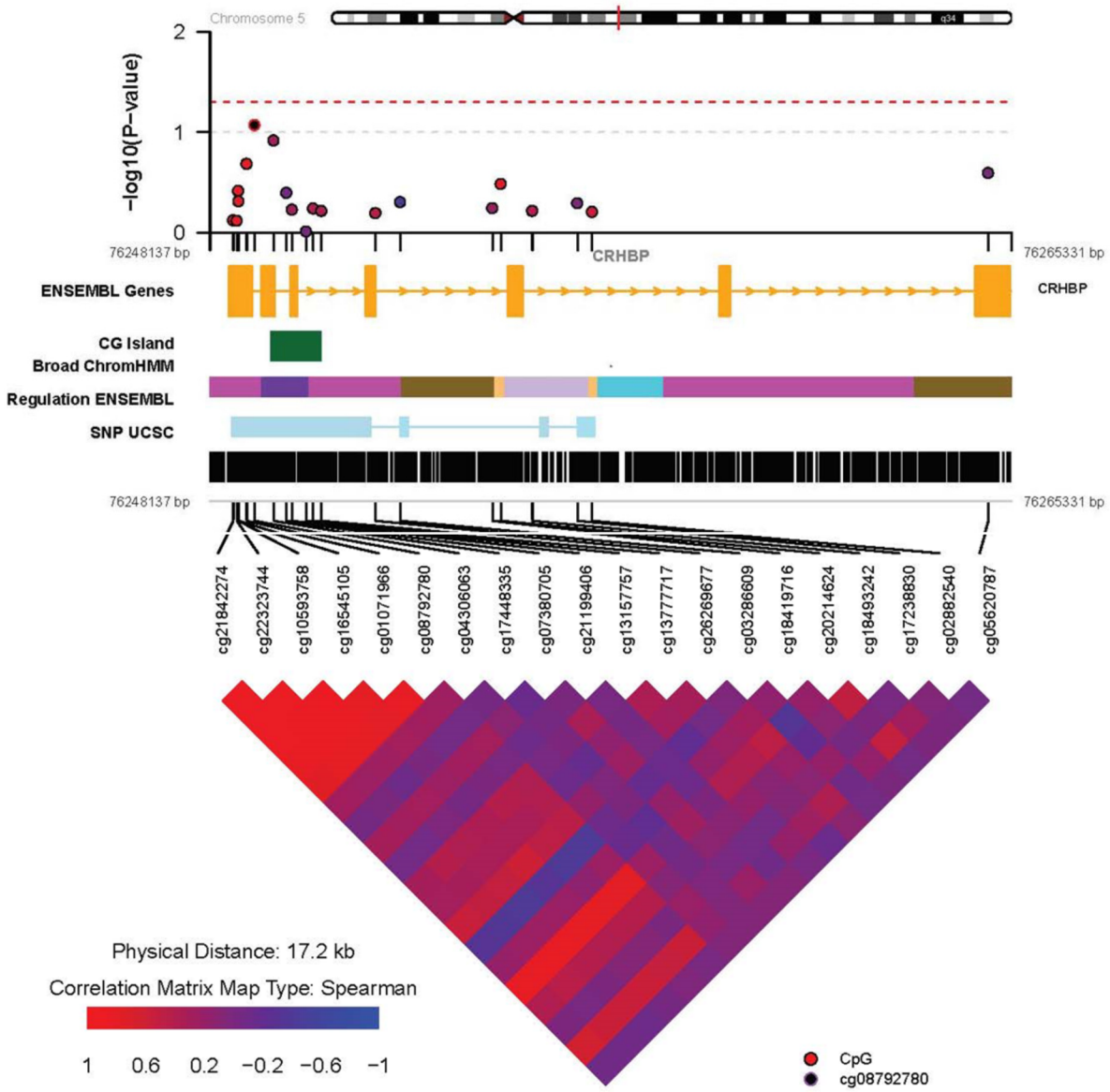
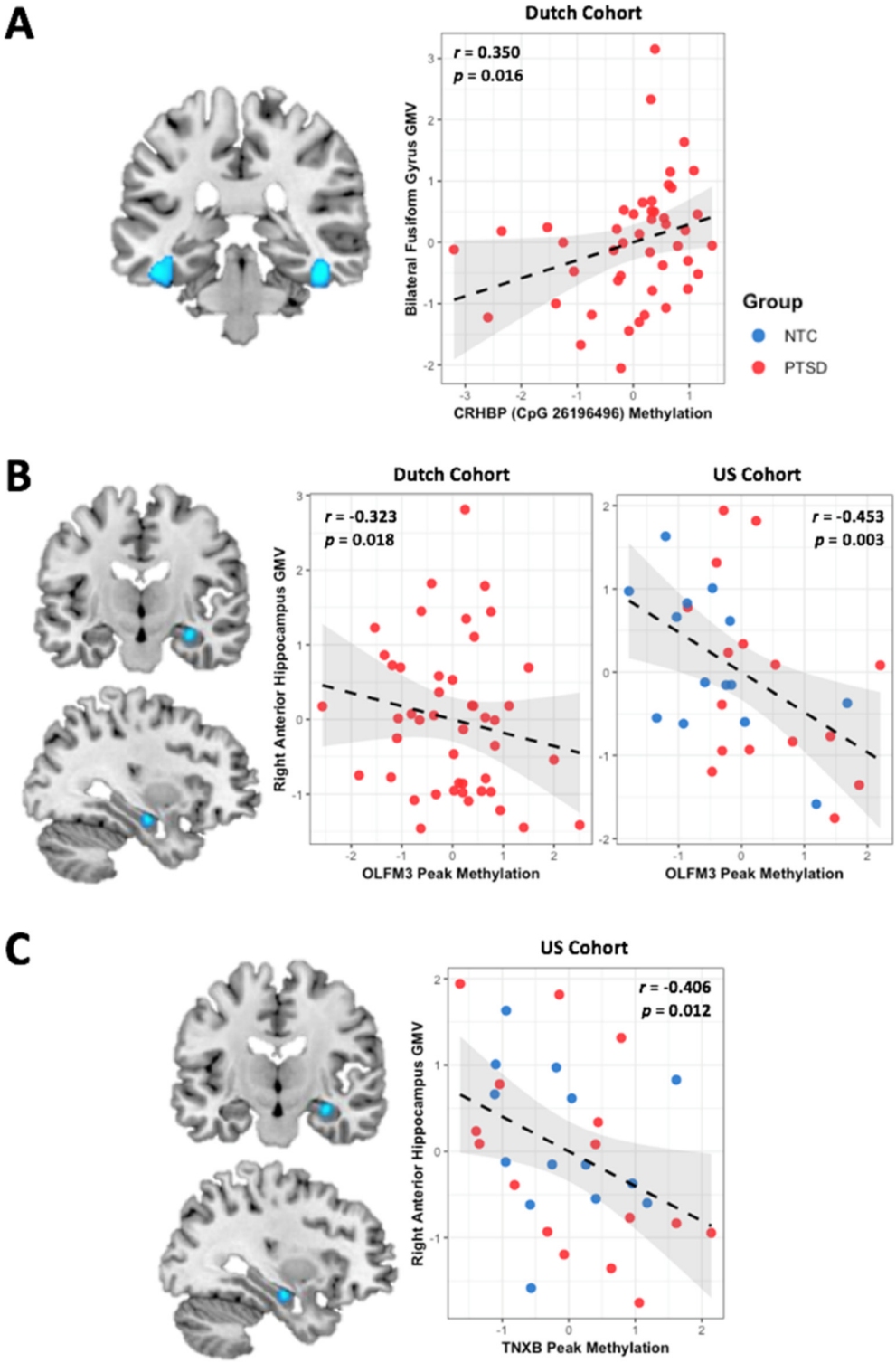


Figure S2: A) Positive correlation between CRHBP and bilateral fusiform gyros in the Dutch cohort B) negative correlation between OLFM3 and the Right Anterior Hippocampus in both cohort C) Negative correlation TNXBmethylation with the Anterior Hippocampus in the USA Cohort.



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