Research Article

METHYLATION OF SEROTONIN RECEPTOR 3A IN ADHD, BORDERLINE PERSONALITY, AND BIPOLAR DISORDERS: LINK WITH SEVERITY OF THE DISORDERS AND CHILDHOOD MALTREATMENT

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Background: *Serotonin 3A receptor (5-HT$_{3A}$R) is associated at the genetic and epigenetic levels with a variety of psychiatric disorders and interacts with early-life stress such as childhood maltreatment. We studied the impact of childhood maltreatment on the methylation status of the 5-HT$_{3A}$R and its association with clinical severity outcomes in relation with a functional genetic polymorphism.* **Methods:** *Clinical severity indexes of 346 bipolar, borderline personality, and adult attention deficit hyperactivity disorders patients were tested for association with the DNA methylation status of eight 5-HT$_{3A}$R gene CpGs. Relationship between the functional variant rs1062613 (C > T) and methylation status on severity of the disorders were also assessed.* **Results:** *Childhood maltreatment was associated with higher severity of the disease (higher number of mood episodes, history of suicide attempts, hospitalization, and younger age at onset) across disorders and within each individual disorder. This effect was mediated by two 5-HT$_{3A}$R CpGs. Compared to T allele carriers, CC carriers had higher methylation status at one CpG located 1 bp upstream of this variant.* **Conclusions:** *This study shows that epigenetic modification of the 5-HT$_{3A}$R is involved in the mechanism underlying the relationship between maltreatment in childhood and the severity of several psychiatric disorders in adulthood. Depression and Anxiety 33:45–55, 2016.*

Key words: *depression; epigenetics; rs1062613; serotonin receptor; methylation; mood episodes*

INTRODUCTION

Epigenetics recently offered us a new and powerful way to explore biological mechanisms underlying neuropsychiatric disorders. Among epigenetic processes, changes

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Contract grant sponsor: Swiss National Foundation (SNF); Contract grant number: 31003A_155896; Contract grant sponsor: SNF NCCR Synapsy grant.

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Received for publication 7 April 2015; Revised 28 July 2015; Accepted 2 August 2015

DOI 10.1002/da.22406
Published online 9 September 2015 in Wiley Online Library (wileyonlinelibrary.com).

in DNA methylation occurring at specific CpGs have been associated with early-life stress, disease status, and severity of psychiatric disorders.[1–3] For instance, several studies found distinct epigenetic profiles in psychiatric patients according to the presence or absence of exposure to childhood abuse.[1,3,4] Considering the severity of psychiatric disorders and specifically of mood disorders, a recent report showed that stratification for disease severity may increase the statistical power for detecting disease-related epigenetic modifications.[5]

Among the promising genes that could determine vulnerability to psychiatric diseases and their interaction with childhood adversities, the serotonin 3A receptor (*5-HT$_{3A}$R*) is of particular interest. The *5-HT$_{3A}$R* regulates several cellular processes involved in the formation of cortical circuits, including interneuron migration and pyramidal neuron dendritic morphology.[6,7] The *5-HT$_{3A}$R* has recently been shown to be required for exercise-induced neurogenesis and antidepressant effects.[8] At a behavioral level, *Ht$_{3a}$r*-ko mice have been shown to display altered physiological responses to stressors, impairments in social transmission of food preference, and fear extinction.[9–11] Chronic ethanol exposure in adult mice induces DNA methylation changes in the promoter region of the *5-HT$_{3A}$R* gene in blood DNA and in specific brain regions including the hippocampus and prefrontal cortex.[12] These observations in mice were recently supported in human blood DNA where one CpG within the promoter region of *5HT$_{3A}$R* showed significant methylation alterations in alcohol dependence cases.[13] In addition, several studies indicate that alcohol dependence and its treatment may be predicted by genetic variants within the *5HT$_{3A}$R*.[14–16] In humans, genetic studies have also found association between a functional single-nucleotide polymorphism (SNP; rs1062613) in *5-HT$_{3A}$R* and bipolar disorder (BD).[17,18] Interestingly, early-life trauma has been shown to interact with rs1062613 to modulate emotional networks and increase depressive-related symptoms.[19] Taken together, these studies add support to the involvement of *5-HT$_{3A}$Rs* in psychiatric diseases and open the possibility that early-life adversity such as childhood maltreatment could modify the methylation status of *5HT$_{3A}$R* in an allele-specific manner.

Here, we aimed at investigating the methylation profile of several CpGs located within or upstream the *5HT$_{3A}$R* gene in adult subjects suffering from BD, borderline personality disorder (BPD), and attention deficit hyperactivity disorder (ADHD) for which history of childhood maltreatment is known and for which several indicators of the severity of the disease are available. ADHD, BPD, and BD are highly comorbid disorders and share several clinical features among which impulsivity, mood liability, irritability, and interpersonal difficulties.[20] BPD and BD are encountered more often than expected by chance in ADHD subjects and ADHD is often found in the childhood of BPD and BD subjects.[20–28] The overlap between BPD and BD is also considerable as the two disorders are even sometimes difficult to distinguish one from the other.[29–31] These suggest not only that these disorders share common genetic and environmental risk factors but also that the presence of one disorder increases the risk of developing the other.[32–37] From the environmental point of view, exposure to maltreatment in childhood is considered as an important risk factor for the development of these three clinical entities and is associated with unfavorable course and outcomes among individuals suffering from these disorders.[38–45] At the genetic level, mounting evidence suggest that neuropsychiatric disorders share overlapping sets of common genetic risk factors and more specifically genes implicated or expressed in the developing human brain including serotonin receptors such as 5HT$_{3A}$R.[46–52] Based on these evidences, we hypothesized that childhood maltreatment and *5HT$_{3A}$R* methylation status will be associated with the indicators of the severity of the disease. We also hypothesized that *5HT$_{3A}$R* methylation status will mediate the effect of childhood maltreatment on adulthood psychopathology. In addition, as rs1062613 was shown to be associated with BD and to interact with early-life stress, we further hypothesized a modulation of our findings by this functional SNP.

METHODS

SUBJECTS

The sample consisted of three different groups of patients all recruited in a specialized outpatient center for the treatment and care of patients suffering from BD, BPD, and adult ADHD. The assessment of BPD and ADHD subjects has previously been described[1,19,53,54] and briefly consisted of the diagnostic interview for genetic studies providing lifetime DSM-IV Axis I disorders.[55] In addition, BPD subjects were assessed using the Structured Clinical Interview for DSM-IV (SCID-II) BPD part questionnaire and only those with a score of 5 or above were recruited in the framework of this study.[56] For ADHD subjects, a diagnosis of ADHD was made upon a careful clinical interview searching for specific attentional and/or impulsive/hyperactive symptoms according to DSM-IV/V criteria. In addition, the existence of the disorder before age of 12 was required for ADHD subjects. ADHD subjects also completed the Adult ADHD Self-Report Scale (ASRS v1.1) and the Wender Utah ADHD Rating Scale (WURS).[57–59] The Diagnostic Interview for ADHD in adults (DIVA 2.0) was used to ascertain the number of symptoms in cases for which a doubt concerning the diagnosis persisted after the clinical evaluation.[60] BD subjects were evaluated with both a clinical interview and the SCID I.[61] Diagnosis of BD was retained only after a best estimate procedure with a senior psychiatrist trained in assessing BD.

For all subjects, history of childhood maltreatment was assessed using the Childhood Trauma Questionnaire (CTQ) which evaluates severity of five types of trauma on the basis of a self-report questionnaire: sexual abuse, physical abuse and neglect, emotional abuse and neglect. A total score is also provided. Although Bernstein and Fink[62] originally suggested to use categories for the different types of maltreatment, several recent studies used them as continuous outcomes and we therefore decided to do so in the following analyses.

All subjects were from European ancestry, gave their written informed consent, and the study was approved by the ethical committee of the Republic and Canton of Geneva.

INDEXES OF SEVERITY

The following variables were used as indicative of the severity of the disorder: (1) history of suicide attempt as it has been shown to be a good predictor of poor treatment response and poor economical and socioeducational status,[63] (2) previous hospitalization and (3) number of hospitalization (only available for BPD and ADHD subjects) as they strongly reflect the impairment related to the disorder, and (4) lifetime comorbid substance use disorder and (5) lifetime comorbid alcohol dependence as they have not only been associated with poor outcomes but also, for the latter, with different levels of *5HT$_{3A}$R* methylation status.[12,13,64] In addition, for subjects having a mood disorder, number of mood episodes, age at onset of mood disorder, and mood episodes with psychotic symptoms were also selected as indexes of disorder severity as they have previously been shown to be good indicators of a disorder's outcome.[63,65,66]

5HT$_{3A}$R METHYLATION ASSESSMENT

DNA Extraction and Bisulfite Conversion. Genomic DNA was extracted from blood samples using the Illustra Nucleon Genomic DNA Extraction kit (GE Healthcare, Dubendorf, Switzerland). Bisulfite conversion of unmethylated cytosines to uracil was performed on 1 μg of DNA using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) and eluted in a final volume of 20 μl EB Buffer. Samples were then stored at −20°C.

Polymerase chain reaction (PCR) Amplification and Pyrosequencing. Each 15 μl final volume of PCR reactions contains 1 μl of bisulfite-modified DNA (100 ng/μl), 1 μl of assay-specific forward and 1 μl of biotinylated reverse primers (10 pMol/μl each) are listed in Supporting Information Table S1, 7.5 μl of HotStarTaq Master Mix (Qiagen, ref. no. 203443) and 4.5 μl water. Thermal Polymerase chain reaction (PCR) conditions were as follows: 95°C for 15 min followed by 50 cycles of 95°C for 30 s, 50.4°C (Pyro_II and Pyro_III) or 61°C (Pyro_I) for 30 s, 72°C for 10 s. Optimal annealing temperature was determined experimentally by using a temperature gradient. A no-template negative control was added to each PCR plate and 1% agarose gel electrophoresis was performed to control for nonspecific amplification.

Five different dilutions (0, 25, 50, 75, and 100%) reconstituted from the unmethylated (0%) and methylated (100%) genomic DNA standards (EpiTect PCR Control DNA, Qiagen) were used as templates to control for correct methylation measurements of each CpG site tested in each assay. For Pyro_I, only the 0, 50, and 100% were tested. PCR products were immobilized onto streptavidin-coated sepharose beads (Fisher Scientific, Reinach, Switzerland, ref[17]-5113-01) using a Vacuum preparation tool (Biotage, Uppsala, Sweden), washed in ethanol 70%, and denatured in 0.2 M NaOH. Single-stranded DNA were then washed in 10 mM Tris-acetate pH 7.6 before being dispensed into 20 mM Tris, 2 mM $MgAc_2$ containing 15 pmol of the assay-specific sequencing primers in 40 μl final volume by gently agitating the aspirator. The resulting single-stranded DNA molecules were then sequenced in a PSQ 96 MA (Qiagen) using appropriate enzymes, substrates, and nucleotides (PyroMark Gold Q96 SQA Reagents, Qiagen) using the nucleotide dispensation orders. The analyzed sequence is shown in Supporting Information Table S1. Each sample was analyzed in duplicates and the recorded methylation difference between the duplicates was set at a threshold of less than 5% for each CpG site in order to control for technical variation.

Originally, 11 CpGs were tested (Fig. 1), but only eight of them showed less than 5% of variation between duplicates and were further investigated in the current study: one CpG within the glucocorticoid response element (GRE; CpG1_I), two in the *5HT$_{3A}$R* promoter region (CpG2_II and CpG3_II), three within the 5′UTR (CpG1_III, CpG2_III, and CpG3_III), and two within the coding sequence (CpG4_III and CpG5_III). The GRE element located upstream of *5HT$_{3A}$R* was identified by Matrix similarity predictions using bio-informatics and functionally tested in electrophoretic mobility shift assay by others.[67]

SNP Characterization of rs1062613. Genomic DNA extracted from blood samples were subjected to PCR amplification using forward primer 5′- biotin- AGGCTGGCTGGGACATGA – 3′ and reverse primer 5′- ATAGGAGTGCCTCCGAGTGCT – 3′ resulting in an 85 bp DNA product corresponding to the sequence located in chromosome 11 at coordinates ranging from 113,975,225 to 113,975,309 on the hg18 human genome version. Each PCR reaction was performed in 25 μl final volume containing 1 μl of 100 ng/μl DNA, 0.5 μl of the biotinylated forward and the reverse primers (20 pMol/μl each), 12.5 μl of HotStarTaq Master Mix (Qiagen, ref. no. 203443), and 10.5 μl of water.

Two-step thermal PCR conditions were as follows: 95°C for 5 min followed by 10 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 20 s and then 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s . A no-template negative control was added to each PCR plate and a 2% agarose gel electrophoresis was performed to control for nonspecific amplification.

PCR products were immobilized onto streptavidin-coated sepharose beads (Fisher Scientific, ref[17]-5113-01) using a Vacuum preparation tool (Biotage), washed in ethanol 70%, and denatured in 0.2 M NaOH. Single-stranded DNA were then washed in 10 mM Tris-acetate pH 7.6 before being dispensed into 20 mM Tris, 2 mM MgAc2 containing 15 pmol of the sequencing primer 5′- CCTCCGAGTGCTCAG – 3′. The resulting single-stranded DNA molecules were then sequenced in a PSQ 96 MA instrument (Qiagen) with the PSQ 96 MA 2.1 software and using appropriate enzymes, substrates, and nucleotides (PyroMark Gold Q96 SQA Reagents, Qiagen). The sequence to analyze was GA/GCGAGGCCCACCAAGGGCCAGCTTGC using the nucleotide dispensation orders TGATCGAGC. The rs1062613 SNP was genotyped automatically by the software based on the relative heights of both peaks generated at positions 2 and 3 written A/G on the sequence to analyze.

Statistical Analyses. Between-group differences in demographic and clinical variables were assessed using linear (for continuous outcomes) and logistic regression (for categorical outcomes) adjusted on age and gender using the "testparm" command in STATA v12.1. Continuous variables were standardized with a Z-transformation. Linear regression models were used in order to assess association between methylation status and childhood maltreatment. Logistic and linear regression models with adjustment on age, gender, and category of diagnosis were used to assess association between clinical variables and CTQ total score and its subscales. Finally, association between *5-HT$_{3A}$R* methylation status and clinical severity variables were assessed using the same models.

A mediation analysis was done when one of the severity variables was associated with both childhood maltreatment and methylation status and when in addition there was an association between same type of childhood maltreatment and methylation status at the same CpG site (an alpha level of $P < .05$ was considered for the selection of the variables at this stage). The methods described by Hicks and Tingley using the "medeff" with 1,000 simulations and 1,000 bootstraps as implemented in STATA v.12.1 was used.[68]

As several tests were performed, a correction for multiple testing was required. Methylation levels at individual CpGs within one pyrosequencing run were highly correlated and thus three independent tests (one for each pyrosequencing run) were considered. For history of childhood maltreatments, CTQ total score and its subscales were tested in association with either CpGs or clinical variables. Three independent tests were also considered due to a high level of correlation between the different types of maltreatment. Six indexes of severity were considered. As they were highly correlated as well only three

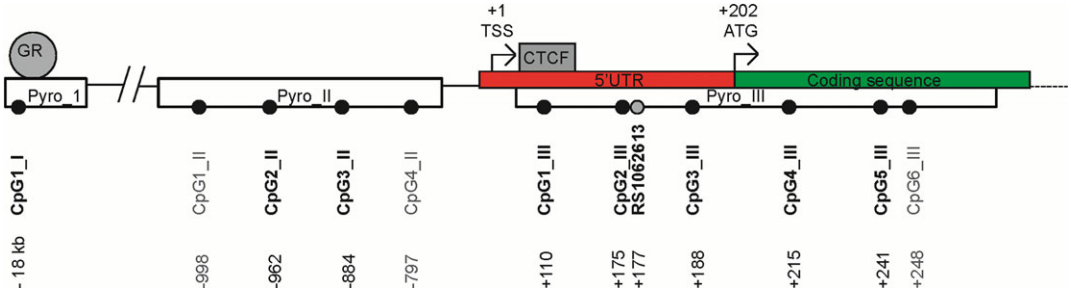

Figure 1. (1) TSS: transcription start site (TSS) of *HTR3AR* located in chromosome 11 at position 113,845,827 in the hg19 human genome version. TSS is located 86 bp before Pyro_III. (2) 5′ UTR: 5′ untranslated region is located in chromosome 11 at positions 113,845,796–113,846,028 (232 bp). (3) ATG: ATG translational start is located in positions 113,846,029–113,846,031. (4) rs1062613: located at position 113,846,005 and consist of a C/T polymorphism. (5) Pyro_III located between positions 113,845,915–113,846,109 and contains 195 bp allowing the analysis of CpG methylation in six CpG sites named CpG1_III to CpG6_III in respect to their position in the obtained pyrogram. The ATG translational start is located 11 bp after CpG3_III and 11 bp before CpG4_III. rs1062613 is just after the guanine of the CpG2_III. The CpG2_III corresponds to cg20621129 that was significantly more methylated in African Americans alcohol-dependent compared with control. [13] Pyro_II is 253 bp in size located 773 bp before the TSS at coordinates 113,844,802–113,845,054. It contains four CpG sites named CpG1_II to CpG4_II in respect to their position in the obtained pyrogram. CpG4_II corresponds to cg08989585 in the goldengate array that was significantly more methylated in alcohol-dependent European Americans compared with controls. [13] (6) Pyro_I: the 82 bp amplified pyrosequencing assay is localized at coordinates 113,827,877–113,827,958. That element contains one site CpG1_I where the guanine residue corresponds to the first base of the GTGGGCCCAGCTGTCCTGG putative glucocorticoid responsive sequence determined previously. [67] (7) CTCF-binding site; positions: chr11:113845935–113845949, Motif Strand: 15 bp = GCCACGAGAGGCAGG. CG is the CpG1_pyroIII. (8) CEBPB-binding sites (not shown); positions: chr11:113845024–113845037, Motif Strand: GATTGCGCCACTGC. CG is CpG4_II.

independent tests were also considered (see Supporting Information Table S2 which displays the degree of correlations for the variables considered in the analyses). Thus, for a correction for multiple testing $P = .05/(3 \times 3 \times 3) = .002$ was considered as a threshold for significance. Nevertheless, all results reaching P value $< .05$ were reported as results of interest and will be discussed accordingly.

RESULTS

The three clinical samples significantly differed in most of the clinical and demographic characteristics (Table 1). Not surprisingly, BPD patients suffered from more severe childhood maltreatment regarding all the subscales of the CTQ compared to the two other groups of patient. Concerning mood disorders, BPD patients had essentially a younger age at onset of mood disorder but a lower number of mood episodes than BD patients. BPD patients distinguished themselves from the two other groups by reporting more often a history of suicide attempt, a higher frequency of lifetime substance and alcohol dependence compared to ADHD and BD subjects. BD subjects had more often a history of psychotic symptoms during a mood episode than the two other clinical groups. The three clinical groups differed significantly in most of the CpG sites (Fig. 2 and Supplementary Material). The SNP rs1062613 was significantly associated with CpG2_III ($b = .69$; $P = 4.23 \times 10^{-7}$), with higher methylation status in CC carriers than T allele carriers (Table 3).

EFFECT OF CHILDHOOD MALTREATMENT ON SEVERITY INDEXES

As obvious from Table 2, after adjustment on age, gender, and category of diagnosis, history of childhood maltreatment was associated with a higher severity of the disorder mainly characterized by: (considering total CTQ) reporting more often a history of suicide attempt (OR = 1.61; $P = 2.7 \times 10^{-5}$), and having at least been hospitalized once (OR = 1.82; $P = 3.5 \times 10^{-5}$). In addition, childhood maltreatment was associated with higher number of mood episodes ($b = .16$; $P = .002$) and younger age at onset of the disorder ($b = -.15$; $P = .002$). Similar types of associations were found when considering the different CTQ subscales and when looking at each individual disorder separately (Table 2 and Supporting Information Table S3).

5HT3AR METHYLATION STATUS AND rs1062613 ACCORDING TO HISTORY OF CHILDHOOD MALTREATMENT

Among the types of childhood maltreatment, physical abuse showed the strongest associations, with CpG3_II: $b = .18$; $P = .001$, with CpG2_III: $b = .19$; $P = .001$, and with CpG5_III: $b = -.19$; $P = .001$ (Table 3). The methylation level at the CpG located within the GRE showed an inverse correlation mainly with emotional neglect ($b = -.16$; $P = .009$; Table 3).

There was an additive effect when looking at the effect of rs1062613 with a higher level of methylation in CC carriers than T allele carriers when considering CpG2_III, independently of the severity of childhood maltreatment. Patients carrying the CC genotype and reporting the highest severity of childhood maltreatment had the highest level of methylation. Indeed, when looking at the association between methylation status at CpG2_III and history of childhood maltreatment, all

TABLE 1. Clinical and demographic characteristics of the three clinical groups

		BPD ($N = 116$)		ADHD ($N = 111$)		BD ($N = 122$)			
		Mean	SD	Mean	SD	Mean	SD	F (df)	P
Age		31.5	9.74	37.65	10.36	45.25	11.7	49.9 (3/345)	<.0001
CTQ	Total score	57.7	18.3	45.41	13.84	40.54	12.9	18.04 (2/306)	<.0001
	Sexual abuse	9.25	5.9	6.15	2.85	6.37	3.38	4.62 (2/307)	.01
	Physical abuse	8.79	4.46	7.14	3.14	6.79	2.86	8.75 (2/307)	.0002
	Physical neglect	8.92	3.51	7.62	2.82	6.69	2.23	9.29 (2/307)	.0001
	Emotional abuse	15.33	5.52	11.49	5.1	9.72	4.63	14.31 (2/307)	<.0001
	Emotional neglect	15.41	4.78	13.02	4.88	10.86	4.78	15.94 (3/306)	<.0001
Mood disorder	Age at onset overall	16.61	7.38	28.55	11.58	21.72	9.86	11.55 (2/243)	<.0001
	Number of episodes (total)	9.01	13.9	1.82	2.35	16.23	11.7	8.85 (2/231)	.0002
		N	%	N	%	N	%	X^2 (df)	P
Gender	Female	106	91.4	33	29.73	65	53.3	90.82 (2)	<.0001
History of SA		73	68.2	12	12.9	45	36.9	36.69 (2)	<.0001
Mood episode with psychotic symptoms		33	30.6	3	3.57	63	52.1	33.75 (2)	<.0001
Previous hospitalization		84	79.3	19	21.35	-	-	37.02 (1)	<.0001
Substance dependence		67	62	29	32.95	28	23	22.04 (2)	<.0001
Alcohol dependence		65	60.2	24	27.27	24	19.7	32.58 (2)	<.0001
Mood disorder	Major depressive disorder	75	64.7	51	45.9	0	0	49.41 (2)	<.0001
	Bipolar disorder	25	21.6	5	4.5	122	100	245.65 (2)	<.0001
rs1062613	CC (vs. CT + TT)	72	62.1	60	54.05	71	62.3	2.05 (2)	.359

statistical models were significantly better (Likelihood Ratio test: $P < .0001$) when adding rs1062613; Supporting Information Fig. S3). No association between rs1062613 and history of childhood maltreatment were found.

5HT$_{3A}$R METHYLATION STATUS ACCORDING TO SEVERITY OF DISORDER

Table 4 shows the association between indices of disorder severity and methylation levels at individual CpGs. Considering CpG1_I in the GRE region, several variables including history of suicide attempt (OR = 0.67; $P = .003$), previous hospitalization (OR = 0.70; $P = .05$), and alcohol dependence (OR = 0.74; $P = .03$) were associated with methylation status at this site. There was an association between CpG3_II and substance dependence (OR = 1.37; $P = .02$). The following variables were associated with higher methylation status of CpG2_III and CpG4_III: history of suicide attempt (OR = 1.75; $P = 4.4 \times 10^{-5}$ and OR = 1.33; $P = .03$), previous hospitalization (OR = 1.84; $P = 5 \times 10^{-4}$ and OR = 1.63; $P = .004$), and lifetime substance dependence (OR = 1.29; $P = .05$ and OR = 1.51; $P = .002$). For CpG5_III, history of suicide attempt (OR = 0.68; $P = .004$), previous hospitalization (OR = 0.60; $P = .001$), and lifetime substance dependence (OR = 0.67; $P = .002$) were all associated with lower methylation status. In addition, CpG5_III methylation level positively correlated with number of mood episodes ($b = .14$; $P = .04$).

MEDIATION ANALYSIS

When considering the severity variables that were associated with both childhood maltreatment and methylation status and when in addition there was an association between the same type of childhood maltreatment and the methylation status at the same CpG site, the only significant mediating effects were those involving childhood physical abuse and CpG2_III and CpG5_III on the following outcomes: history of suicide attempt, previous hospitalization, and number of mood episodes (Fig. 3).

DISCUSSION

The 5-HT$_{3A}$R is a promising candidate for the understanding of early-life vulnerability to psychiatric disorders as it has been shown to be required for cellular events involved in cortical circuit formation[6,7] and interact with early-life adverse events.[19,69] Our results further support this idea by showing that, at an epigenetic level, differential methylation status is observed

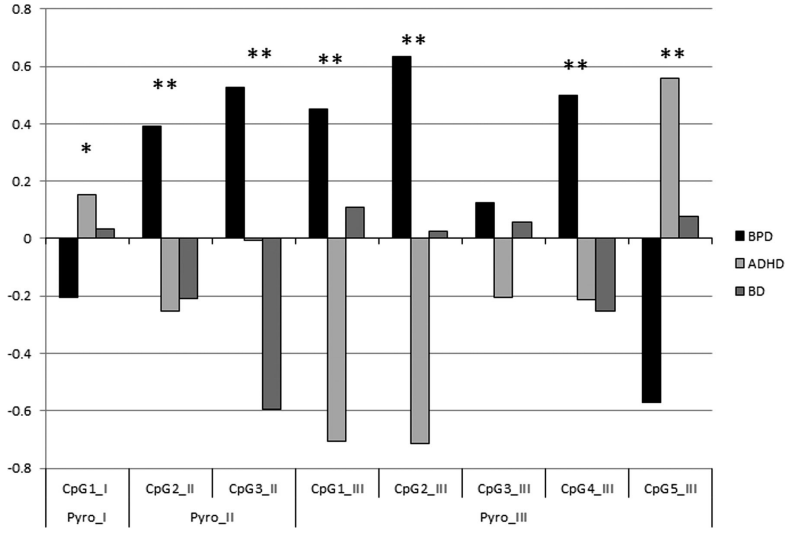

Figure 2. Standardized values of methylation of $5HT_{3A}R$ at individual CpGs according to the three clinical groups (BPD, BD, and ADHD subjects). *$P < .05$; **$P < .001$.

in psychiatric subjects depending on their history of childhood maltreatment and the clinical severity of their disorder.

First and concordant with several studies in the field,[38–45] we showed that childhood maltreatment was associated with more severe expression of psychiatric disorder: higher number of mood episodes, younger age at onset of the disease, hospitalization and history of suicide attempts. This association was moreover not specifically linked to one of the psychiatric disorders we studied (ADHD, BPD, or BD) and was found to be significant even when adding diagnostic category as covariate in the statistical models. This is a strong result which clearly highlights the need to search for history of childhood maltreatment in patients suffering from psychiatric disorders as these events are associated with worse outcome. Second, we found that childhood maltreatment and especially childhood physical abuse had a broad impact on *5-$HT_{3A}R$* methylation levels and that these epigenetics changes might have an impact on the severity of the disorder namely the number of mood episodes, history of suicide attempts, and previous hospitalization. We also found that childhood emotional neglect was inversely correlated with CpG1_I methylation level. This CpG1_I is located within a GRE element upstream of the *5-$HT_{3A}R$* and could thus provide a mechanistic link between stress-induced glucocorticoid levels and the *5-$HT_{3A}R$* gene.[67] Whether early-life adversity modifies the methylation status of the *5-$HT_{3A}R$* in specific brain regions and changes its mRNA expression levels remains to be investigated.

Third, the methylation status of *5-$HT_{3A}R$* was found to be associated with several psychiatric severity outcomes. Indeed, recent findings in the field indicate that considering clinical severity of a given disorder gives power in detecting significant associations. For instance,

TABLE 2. Effect of childhood maltreatment and indexes of severity of the disorder

	Total CTQ		Sexual abuse		Physical abuse		Physical neglect		Emotional abuse		Emotional neglect	
	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*
History of suicide attempt ($N = 305$)	***1.61***	***2.7 × 10⁻⁵***	***1.46***	***.0005***	***1.38***	***.001***	***1.44***	***.0007***	***1.37***	***.001***	***1.45***	***.0008***
Psychotic symptoms ($N = 226$)	1.11	.37	***1.24***	***.05***	1.06	.59	1.09	.47	1.11	.39	0.93	.57
Hospitalization ($N = 208$)	***1.82***	***3.5 × 10⁻⁵***	***2.31***	***.0001***	***1.38***	***.01***	***1.52***	***.002***	***1.59***	***.001***	***1.39***	***.012***
Substance dependence ($N = 303$)	1.15	.18	1.02	.78	1.04	.637	***1.23***	***.043***	1.17	.144	1.09	.366
Alcohol dependence ($N = 303$)	***1.22***	***.05***	1.15	.152	1.07	.439	1.21	.06	***1.26***	***.034***	1.14	.193
	b	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*
Age at onset of mood disorder ($N = 234$)	***−.15***	***.002***	−.04	.28	−.02	.56	***−.11***	***.016***	***−.17***	***.0003***	***−.11***	***.015***
Number of mood episodes ($N = 220$)	***.16***	***.002***	***.16***	***.005***	***.18***	***.003***	.07	.145	***.13***	***.02***	.09	.09

P values are adjusted on age, gender, and diagnostic category. Results reaching a *P* value < .05 are bolded and italicized; those reaching the significant threshold accounting for multiple testing of $P \leq .002$ are underlined. First part of the table displays the results from the logistic regressions for categorical variables; the second part displays the results from linear regression for the continuous variables.

TABLE 3. Effect of childhood maltreatment on *5HT$_{3A}$R* methylation status at individual CpGs

	CTQ_tot		Sexual abuse		Physical abuse		Physical neglect		Emotional abuse		Emotional neglect		SNP	
	b	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*B*	*P*	*b*	*P*
CpG1_I	***−.15***	***.01***	*−.08*	.17	−.09	.13	−.11	.06	−.11	.07	***−.16***	***.009***	.02	.89
CpG2_II	.03	.56	.08	.21	***.13***	***.01***	−.02	.74	.02	.77	−.08	.22	.10	.38
CpG3_II	.10	.09	.08	.23	***.18***	***.001***	−.01	.84	.08	.18	.05	.48	−.01	.98
CpG1_III	−.09	.15	−.01	.89	−.03	.63	−.09	.12	−.09	.10	−.09	.14	−.08	.51
CpG2_III	***.11***	***.05***	.06	.31	***.19***	***.001***	.04	.44	.09	.12	.06	.40	***.69***	***4.23 × 10^{-7}***
CpG3_III	−.01	.83	.06	.33	−.08	.20	−.03	.61	.01	.87	−.01	.82	−.02	.89
CpG4_III	.07	.26	.10	.07	.09	.11	−.01	.86	.08	.15	−.02	.76	.03	.77
CpG5_III	−.09	.08	−.03	.56	***−.19***	***.001***	−.11	.07	−.01	.80	−.08	.14	−.03	.79

Associations between *5HT$_{3A}$R* methylation status and rs1062613 are also displayed. Results reaching a $P \leq .05$ are bolded and italicized; those reaching the significant threshold accounting for multiple testing of $P \leq .002$ are underlined. *P* values are adjusted on age and gender.

an association between *NR3C1* methylation status and clinical indexes of severity such as self-injury, previous hospitalization, or current employment was found in a sample of BPD.[2] Using a mediation analysis, we found that a fraction of the association between childhood maltreatment and clinical severity indexes is mediated by *5HT$_{3A}$R* methylation status. Our results also suggest that this is not the case for all type of childhood maltreatment and all kind of severity indexes and all CpGs. This result is in the line of previous findings suggesting that individual CpGs could be more specifically associated with a given type of clinical outcome.[64,70]

Interestingly, one of our severity indexes, namely substance dependence, showed association with individual CpGs. Previous findings have revealed an association between alcohol consumption and changes in *5HT$_{3A}$R* methylation status in mice[12] and humans.[13] More specifically, it was reported that *5HT$_{3A}$R* blood DNA methylation profile appeared to be associated with alcohol dependence with specifically two CpGs cg20621129 (corresponding to our CpG2_III) and cg08989585 (corresponding to our CpG4_III) showing higher methylation status, respectively, in African American alcohol dependents compared with control and in European American alcohol dependents compared with controls.[13] These findings are consistent with our results as we also found higher methylation level of CpG4_III (which is highly correlated with CpG2_III) in subjects reporting substance use disorders. These results thus highlight the need to further investigate *5HT$_{3A}$R* epigenetics in substance abuse (including alcohol) disorders as these environmental factors seem to leave an epigenetic signature on this gene.

An intriguing result was the modulation of *5HT$_{3A}$R* methylation status at CpG2_III by rs1062613. Allele-specific modulation of CpG methylation has been previously reported in the context of early-life adversity and psychiatric disorders.[71] More specifically, *FKBP5* DNA demethylation in subjects exposed to childhood trauma was found to be specific for a previously validated risk allele.[71] In our study, we found evidence that the functional *5HT$_{3A}$R* SNP (rs1062613) specifically influenced the methylation status of a CpG located at 1 bp of the SNP. We more specifically found that patients carrying the risk CC genotype showed the highest level of methylation at CpG2_III thus leading to an additive effect when considering, for instance, the clinical status or history of childhood maltreatment. Interestingly, the CC genotype has been previously reported to interact with early-life trauma and modulate depression-related symptoms as well as brain networks involved in emotional processing.[19,69] Given that the C allele is functionally linked to lower expression levels of *5HT$_{3A}$R* Mrna,[18] we suggest that increased methylation of the neighboring CpG due to exposure to childhood maltreatment could lead to a further decrease in the expression of *5HT$_{3A}$R* mRNA. Whether stress-induced decreased levels of *5HT$_{3A}$R* mRNA in brain regions are linked to the emergence of psychiatric-relevant phenotypes remains to be further investigated. Taken together, our results reveal a complex interplay between early-life adversity and CpG epigenetic sites located in the vicinity of a functional *5HT$_{3A}$R* SNP.

Our study has several limitations. First, most of our results are driven by BPD subjects, which not only have the higher severity indexes but also report the higher severity of childhood maltreatment. Thus, the observed association might reflect other confounding variables not taken into account in this study and that may be more often found in BPD subjects for instance other environmental factors and/or specific psychopharmacological treatment such as antidepressants, factors which have been shown to modify epigenetic processes.[53][72–74] Nevertheless, as a specialized center usually assessing and receiving hard-to-treat patients, the majority of our patients are under psychotropic medication (see Perroud et al.[53] and Prada et al.[54] for a detailed description of the medications taken by BPD and ADHD subjects in our center) and we thus believe that the impact of such treatment on our findings should be minimal. Moreover, when restricting the analyses to one or the other of the investigated disorders, several of the

TABLE 4. Effect of *5HT$_{3A}$R* methylation status at individual CpGs on variables of severity of the disorder (*P* adjusted on age and genre)

	CpG1_I		CpG2_II		CpG3_II		CpG1_III		CpG2_III		CpG3_III		CpG4_III		CpG5_III	
	b	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*	*b*	*P*
Age at onset of mood disorder	.02	.69	−.08	.20	.08	.24	−.04	.56	.06	.37	.03	.61	.03	.69	−.03	.55
Number of mood episodes	−.09	.15	.02	.71	−.01	.86	.11	.20	***−.17***	***.008***	−.08	.20	−.13	.06	***.14***	***.04***
	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*
History of suicide attempt	***0.67***	***.003***	0.94	.69	1.08	.55	1.27	.15	***1.75***	***4.4 × 10^{-5}***	0.90	.42	***1.33***	***.03***	***0.68***	***.004***
Psychotic symptoms	1.04	.75	0.96	.78	0.78	.93	***1.64***	***.01***	1.01	.92	0.88	.36	0.99	.98	1.01	.89
Hospitalization	***0.70***	***.05***	1.06	.72	1.08	.65	1.29	.20	***1.84***	***5 × 10^{-4}***	1.15	.40	***1.63***	***.004***	***0.60***	***.001***
Substance dependence	0.85	.216	1.07	.60	***1.37***	***.02***	0.79	.14	***1.29***	***.05***	0.92	.55	***1.51***	***.002***	***0.67***	***.002***
Alcohol dependence	***0.74***	***.03***	1.05	.68	1.24	.11	0.80	.17	1.21	.15	0.84	.18	1.16	.23	0.87	.27

Results reaching a $P \leq .05$ are bolded and italicized; those reaching the significant threshold accounting for multiple testing of $P \leq .002$ are underlined.

associations were in the range of those observed in the whole sample, suggesting that our findings are not entirely driven by BPD subjects. A second limitation is related to the fact that methylation analyses were performed on DNA samples of leukocytes and that we did not study brain tissue. In addition, it was thus not possible to assess whether methylation changes were related to a specific subset of white blood cells or whether childhood maltreatment modified the fraction of leukocytes, thus introducing further confounding factors. A third limitation is the self-report nature of the childhood maltreatment, which may have affected our results by recollection bias. Fourthly, we decided to report and discuss all results reaching a *P* value < .05 and thus some of them are not significant after correction for multiple testing. Nevertheless, even with a very stringent correction for multiple tests, several of our findings specially the most interesting ones such as the effect of rs1062613 on CpG2_III, the association between clinical status and methylation levels, or the mediation analyses remained significant. We are thus quite confident that our findings reflect true associations and not false-positive findings. Additionally, the different assessment of subjects across disorders might also have influenced our data. Finally, our study lacks a control group, which would have allowed us to assess *5HT$_{3A}$R* methylation levels in individuals without severe psychiatric disorders. Although this constitutes a limitation, we believe that the cross-disorder strategy of our study is well designed to measure the association between *5HT$_{3A}$R* methylation and indexes of psychopathology across disorders. Nevertheless, further studies should investigate differences in *5HT$_{3A}$R* methylation status in psychiatric cases and controls in order to determine whether changes in *5HT$_{3A}$R* methylation levels are associated to disease state.

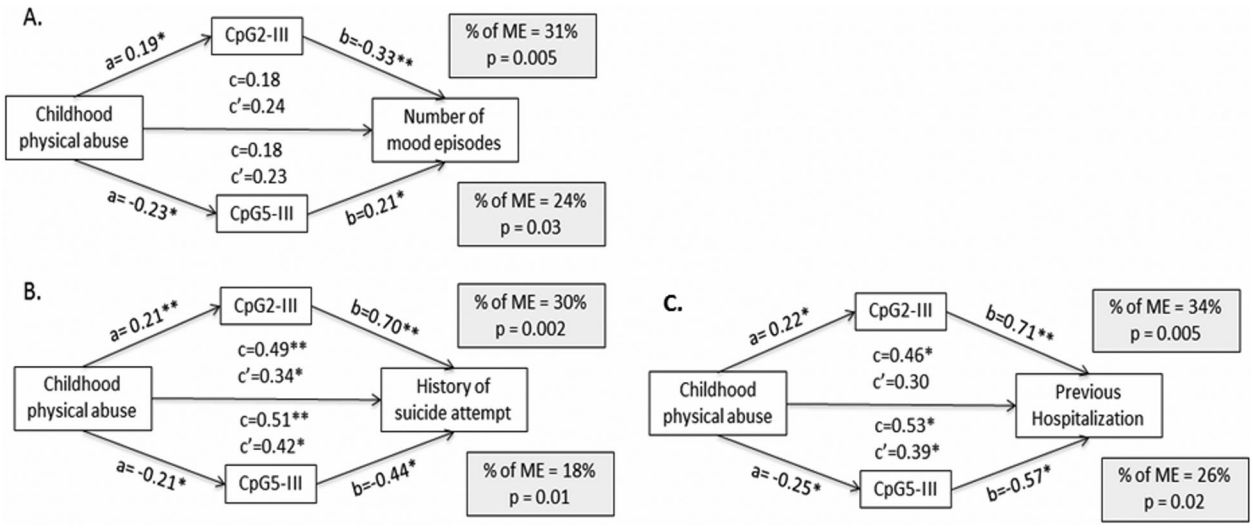

Figure 3. Standardized path coefficients of the mediation model of valence of childhood physical abuse on number of mood episodes (A), history of suicide attempt (B), and previous hospitalization (C) through methylation level of CpG2_III and CpG5_III. *c*, total effect of physical abuse on the outcomes (*ab* + *c'*); *c'*, direct effect of physical abuse on outcomes; *a*, effect of the independent variable on the mediator; *b*, effect of the mediator on the dependent variable. **P* < .01; ***P* < .001.

CONCLUSIONS

In conclusion, our study reveals a complex interplay between early-life adversity, CpG sites located in the vicinity of a functional *5HT$_{3A}$R* SNP and clinical severity outcomes. More generally, it supports the hypothesis that the *5HT$_{3A}$R* gene may be a target for early-life adversity and could modulate the emergence and severity of psychiatric phenotypes in adulthood.

Acknowledgments. Work in the Dayer laboratory was supported by a Swiss National Foundation (SNF) grant (31003A_155896) and the SNF NCCR Synapsy grant.

Conflict of interest and/or financial disclosures: N.P. has received honoraria for participating in expert panels and conferences from Lundbeck and Elli-Lilly. J.-M.A. has received honoraria for participating in expert panels and conferences from Lundbeck, Elli-Lilly, Astra, Servier, Ostuka, Janssen, and Vifor. There is no conflict of interest in relation to this article.

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