SHORT REPORT

DNA Methylation Analysis of *HTR2A* Regulatory Region in Leukocytes of Autistic Subjects

Dubravka Hranilovic, Sofia Blazevic, Jasminka Stefulj, and Peter Zill

Disturbed brain and peripheral serotonin homeostasis is often found in subjects with autism spectrum disorder (ASD). The role of the serotonin receptor 2A (HTR2A) in the regulation of central and peripheral serotonin homeostasis, as well as its altered expression in autistic subjects, have implicated the *HTR2A* gene as a major candidate for the serotonin disturbance seen in autism. Several studies, yielding so far inconclusive results, have attempted to associate autism with a functional SNP −1438 G/A (rs6311) in the *HTR2A* promoter region, while possible contribution of epigenetic mechanisms, such as DNA methylation, to HTR2A dysregulation in autism has not yet been investigated. In this study, we compared the mean DNA methylation within the regulatory region of the *HTR2A* gene between autistic and control subjects. DNA methylation was analysed in peripheral blood leukocytes using bisulfite conversion and sequencing of the *HTR2A* region containing rs6311 polymorphism. Autistic subjects of rs6311 AG genotype displayed higher mean methylation levels within the analysed region than the corresponding controls ($P < 0.05$), while there was no statistically significant difference for AA and GG carriers. Our study provides preliminary evidence for increased *HTR2A* promoter methylation in leukocytes of a portion of adult autistic subjects, indicating that epigenetic mechanisms might contribute to *HTR2A* dysregulation observed in individuals with ASD. ***Autism Res*** *2016, 9: 204–209*. © 2015 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: autism; serotonin; HTR2A; DNA methylation; epigenetics

Introduction

Serotonin (5-hydroxytryptamine, 5HT) is a well-known monoamine implicated in a wide range of physiological processes including cardiovascular regulation, haemostasis, gastrointestinal functions, circadian rhythms, cognition, mood, and social behaviours. Serotonin signalling also participates in the regulation of many neurodevelopmental processes and accumulating evidence suggests that it might play a role in the developmental programming of childhood- and adult-onset mental conditions [Bonnin & Levitt, 2011]. Numerous studies point to disturbed brain and peripheral serotonin homeostasis in subjects with autism spectrum disorder (ASD) [for a review see Hranilovic & Blazevic, 2012]. Several lines of evidence implicated serotonin receptor type 2A (HTR2A) as a possible cause or potential marker of the serotonergic disturbances seen in autism. First, atypical antipsychotics, acting via HTR2A, are known to alleviate repetitive behaviour and aggression in ASD patients [Buitelaar & Willemsen-Swinkels, 2000; Marek, Carpenter, McDougle, & Price, 2003]. Furthermore, ASD subjects or their relatives displayed significant reduction in cortical [Goldberg et al., 2009; Murphy et al., 2006; Oblak, Gibbs, & Blatt, 2013] as well as platelet [Cook et al., 1993; McBride et al., 1989] HTR2A binding. Also, platelet aggregation, an indirect measure of platelet HTR2A activity/number, was found to be reduced in ASD subjects [Hranilovic et al., 2009; McBride et al., 1989; Safai-Kutti, Denfors, Kutti, & Wadenvik, 1988].

It is becoming increasingly evident that epigenetic mechanisms, including DNA methylation of cytosine residues, play a role in the fine-tuning of gene expression and may contribute to the development of neuropsychiatric disorders [Tsankova, Renthal, Kumar, & Nestler, 2007]. DNA methylation of cytosine residues occurs predominantly in the context of CpG dinucleotides which are often grouped in so called CpG islands, a CG-enriched stretches of DNA that typically occur in promoter regions of many genes and are involved in the transcriptional control. Epigenetic mechanisms are also involved in neural development and alterations of DNA methylation have been linked to pathogenesis of several neurodevelopmental disorders, such as Rett

From the Department of Animal Physiology, Division of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia (D.H., S.B.); Laboratory of Neurochemistry and Molecular Neurobiology, Division of Molecular Biology, Rudjer Boskovic Institute, Zagreb, Croatia (J.S.); Catholic University of Croatia, Zagreb, Croatia (J.S.); Division of Psychiatric Genetics and Neurochemistry, Department of Psychiatry and Psychotherapy, Ludwig-Maximilian University of Munich, Munich, Germany (P.Z.)
Jasminka Stefulj and Peter Zill have contributed equally to this work.
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Address for correspondence and reprints: Jasminka Stefulj, Laboratory of Neurochemistry and Molecular Neurobiology, Division of Molecular Biology, Rudjer Boskovic Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia. E-mail: jasminka.stefulj@irb.hr
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syndrome, fragile X syndrome or Parder Willi and Angelman syndromes [Rangasamy, D'Mello, & Narayanan, 2013; Schanen, 2006]. Large-scale DNA methylation profiling of lymphoblast cell lines has indicated altered methylation of CpG islands as an epigenetic contribution also to the development of autism [Nguyen, Rauch, Pfeifer, & Hu, 2010], and changes in the expression of several genes have been observed in autistic subjects as a result of aberrant DNA methylation [Gregory et al., 2009; Nagarajan, Hogart, Gwye, Martin, & Lasalle, 2006; Nguyen et al., 2010]. The *HTR2A* promoter region lacks typical CpG island, but it contains a number of methylated cytosines in the context of CpG dinucleotides [Falkenberg, Gurbaxani, Unger, & Rajeevan, 2011]. In addition, low but appreciable levels of methylation at several non-CpG cytosines in the *HTR2A* promoter region have been also reported [Abdolmaleky et al., 2011]. The methylation pattern of the *HTR2A* promoter was found to be altered in several mental health conditions [Abdolmaleky et al., 2011; Dammann et al., 2011; De Luca, Viggiano, Dhoot, Kennedy, & Wong, 2009; Falkenberg et al., 2011] and was recently associated with neurodevelopmental outcomes in healthy newborn infants [Paquette et al., 2013], but has not yet been analysed in relation to autism [Paquette & Marsit, 2014]. We hypothesized that altered methylation of the *HTR2A* promoter region might be a contributing factor to HTR2A dysregulation observed in autism. Hence, this study was aimed to investigate possible differences in the mean methylation within the *HTR2A* promoter region between autistic patients and healthy control subjects.

Methods

Participants

The study included 90 autistic patients (age range 4–45 years) and 66 healthy control subjects (age range 19–60 years). Studied population characteristics are given in Supporting Information Table 1. Autistic patients were recruited at the Centres for autism Zagreb, Rijeka and Split (Republic of Croatia) and were diagnosed with autism according to DSM-IV criteria. Control subjects were blood donors (recruited at the Croatian Institute of Transfusion Medicine) with no life-time history of mental illnesses, behavioural disorders, or substance abuse. All subjects were part of our previous genetic association study [Hranilovic et al., 2010]. For this study we excluded subjects with insufficient residual DNA or that did not meet the criteria of no life-time history of smoking, as smoking was reported to induce genome-wide changes in DNA methylation status [Zeilinger et al., 2013]. The study was in accord with the Declaration of Helsinki, and was approved by the Ethics Committee of the Medical Faculty of the University of Zagreb. An informed consent was obtained from the control subjects and the patients' parents.

Experimental Procedures

Genomic DNA was isolated from whole blood using the DNA Isolation Kit for Mammalian Blood (Boehringer Manheim, Germany). Uniform amounts of DNA (750 ng) were treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A 383 bp region (−1691/−1308 bp related to the *HTR2A* start codon; NG_013011.1) was amplified from bisulfite modified DNA (75 ng per reaction) by two-step PCR, using the following primers: forward (NG_013011.1: −1691/−1673 bp related to *HTR2A* start codon), 5′-TGG TGG GGG AAA AAA ATT-3′; reverse (NG_013011.1: −1331/−1308 bp related to *HTR2A* start codon), 5′-AAA TAA CCT TTT ATA CAA ATT CCC-3′. Control PCR reaction using the original (unmodified) DNA as a template was also included and yielded no detectable PCR product. PCR products were purified by shrimp alkaline phosphatase (SAP) treatment and subjected to bidirectional Sanger sequencing using BigDye chemistry, ABI 310 capillary sequencer, and Sequencing Analysis version 5.3.1 software (all from Applied Biosystems) as described previously [Zill et al., 2012]. Three partially methylated cytosines were identified, located 1522 bp (chr13: 46,897,427 in GRCh38), 1439 bp (chr13: 46,897,344 in GRCh38) and 1421 bp (chr13: 46,897,326 in GRCh38) upstream from the *HTR2A* start codon. All other cytosines in the region yielded only thymidine signals, indicating perfect bisulfite conversion. The approximate methylation frequency at each methylated site (expressed in %) was calculated by comparing the peak height of the cytosine signal with the sum of the peak heights of cytosine and thymidine signals, as described by Melki, Vincent, & Clark [1999] and validated by Jiang et al. [2010]. rs6306 was genotyped using the TaqMan® SNP Genotyping Assay (Applied Biosystems). The rs6311 genotype was determined in our previous study [Hranilovic et al., 2010] and confirmed here by direct sequencing. Figure 1 shows locations of the three methylation sites assayed in our study, in relation to polymorphisms rs6311 and rs6306.

Statistical Analyses

Results were processed with GraphPad InStat 3.01 and JMPIN software. The normality of the data was tested using the method of Kolmogorov and Smirnov. The equality of variance was tested using Bartlett's test. Genotype and gender effects were assessed by two-way and one-way ANOVA, respectively. Continuous variables were correlated using Pearson's or Spearman's method, as appropriate. Differences between the autistic and control groups were tested using one-way ANOVA or Kruskal–Wallis test followed by Bonferroni's or Dunn's post hoc tests, respectively. The level of significance was set at 0.05.

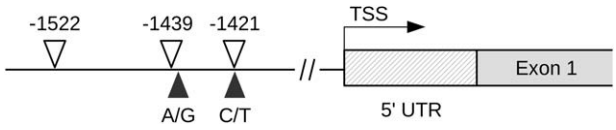

Figure 1. Schematic representation of the three studied methylation sites in *HTR2A* promoter region. Open triangles indicate locations of the analysed methylation sites, while closed triangles indicate locations of the polymorphic sites rs6311 (−1438 A/G) and rs6306 (−1421 C/T). Numbers above open triangles indicate positions in bp relative to the ATG start codon in exon 1. The core transcription start site (TSS) is indicated by the arrow. 5′ UTR–5′ untranslated region.

Results and Discussion

Genotype Dependency and Interloci Correlation of DNA Methylation in the Selected Region

For DNA methylation analysis, we selected the *HTR2A* promoter region encompassing SNPs rs6311 (−1438 G/A) and rs6306 (−1421 C/T), both of which were shown to act as transcription factors binding sites [Falkenberg & Rajeevan, 2010; Smith et al., 2008]. Three partly methylated cytosines were observed in the selected region (Fig. 1). Two of them, located 1421 bp and 1439 bp upstream from the *HTR2A* start codon, have been previously described [Abdolmaleky et al., 2011; Falkenberg et al., 2011], while the one located at −1522 bp corresponds to probe cg10323433 in Infinium HumanMethylation450 BeadChip Kit. The identified loci −1522, −1439, and −1421 were methylated to different extents and with different degrees of variation (mean ± SD): 86.9 ± 3.9%, 57.5 ± 35.3%, and 70.1 ± 17.1%, respectively, in the control sample ($P < 0.0001$, KW = 43.86); 91.4 ± 3.5%, 71.9 ± 31.0%, 78.5 ± 17.1%, respectively, in the autistic sample ($P < 0.0001$, KW = 28.52). Since rs6311 minor allele A leads to CpA instead of CpG dinucleotide at −1439 site, methylation frequency at this position strongly depended on the rs6311 genotype ($P < 0.0001$ in both control and autistic sample). Moreover, rs6311 influenced the methylation frequency also at −1421 and −1522 sites, with rs6311 GG carriers having the highest and AA carriers the lowest methylation levels ($P < 0.0001$ in control and autistic sample for both loci). While these results accord with previously reported findings [Falkenberg et al., 2011], it should be noted that rs6311 exerted opposite effect on the methylation levels in *HTR2A* intragenic region [Smith et al., 2013]. rs6306, leading to loss of −1421 cytosine available for methylation in T allele carriers, strongly influenced methylation status at the −1421 site ($P < 0.0001$ in control and autistic sample), but had no effect on the methylation frequency at either −1522 ($P = 0.808$ and 0.877 in control and autistic sample, respectively) or −1439 ($P = 0.997$ and 0.215 in control and autistic sample, respectively) site. Though rs6306 is generally considered to be a rare DNA variation and its frequency is usually not reported in the methylation studies, a minor allele T frequency in our population was 8.3 and 8.9% in control and autistic group, respectively, similar to UK population [7.8% in controls; Spurlock, Heils, & Holmans, 1998]. Our findings argue for consideration of this SNP in case-control and functional studies of DNA methylation in the respective region. Methylation levels at the three loci were highly correlated with each other ($P < 0.0001$ for all combinations), as anticipated based on the effects of rs6311 and rs6306 genotypes. When subjects were stratified according to rs6311 and rs6306 genotypes, we still found statistically significant positive correlations in subjects with genotype combinations including rs6306 CC genotype, while in subjects with genotype combinations including rs6306 CT genotype, statistical significance of correlations was less obvious (see Supporting Information Table 2), possibly due to smaller sizes of these groups.

Age and Gender Influences on the Mean Methylation Within the Selected Region

Due to genotype dependency of DNA methylation and relatively small number of subjects with rs6306 CT or TT genotypes, we excluded rs6306 T carriers from further analyses and considered the mean methylation of the three methylated loci in rs6306 CC homozygotes (55 control and 75 autistic subjects) stratified according to rs6311 genotype. The mean methylation within the selected region was not affected by gender in either autistic ($P = 0.381$, 0.437 and 0.213 for AA, AG and GG genotype, respectively) or control ($P = 0.425$, 0.127 and 0.368 for AA, AG and GG genotype, respectively) sample, which is consistent with the findings reported by Ghadirivasfi et al. [2011]. On the other hand, there was a negative (in most cases significant) correlation between the mean methylation and age in control ($r = -0.677$, $P < 0.01$ for AA; $r = -0.434$, $P < 0.05$ for AG, and $r = -0.488$, $P = 0.194$ for GG) and autistic ($r = -0.558$, $P < 0.01$ for AA; $r = -0.488$, $P < 0.01$ for AG, and $r = -0.488$, $P < 0.01$ for GG) subjects. A trend towards decrease in *HTR2A* methylation with age was observed also in other studies [Abdolmaleky et al., 2011; Ghadirivasfi et al., 2011; Polesskaya, Aston, & Sokolov, 2006].

Comparison of the Mean Methylation Between Autistic and Control Subjects

Our autistic sample consisted of pre- and post-pubertal individuals, while the control sample included only adults, due to ethical reasons and the possibility to exclude behavioural/mental disorders, symptoms of which appear usually after puberty. To avoid age interference in the comparison of the mean methylation levels between autistic and control subjects, we separated post-

pubertal autistic subjects (19–45 years), age-matched with the control group, from autistic children (4–18 years) (Table 1). The mean methylation levels significantly differed across the three groups ($P = 0.0246$, $F = 4.142$; $P = 0.0003$, KW = 16.007; $P = 0.0053$, KW = 10.483 for GG, AG and AA carriers, respectively). As could be seen in Figure 2, values in patients of rs6311 AG and AA genotype tended towards modest increase as compared to age-matched controls, however statistically significant differences between age-matched groups were observed only for AG ($P < 0.05$, Dunn's post hoc test), but not AA ($P > 0.05$, Dunn's post hoc test) carriers, possibly due to smaller number of AA subjects. At the moment we are not able to explain why no alterations were present in rs6311 GG carriers since we focused our analysis on the mean methylation within the selected region (we consider it a more accurate approach when the number of subjects is limited). This phenomenon should be explored employing individual loci analyses in larger samples.

Multiple studies pointed to altered HTR2A density/activity in the brain and platelets of autistic subjects (see Introduction), but the underlying molecular mechanisms remain elusive. The rs6311 polymorphism might play such a role as it was shown to modulate HTR2A mRNA expression [Smith et al., 2013; Smith, Banks, Hansen, Sadee, & Herman, 2014]. We have previously observed overrepresentation of rs6311 G allele in autistic subjects relative to controls [Hranilovic et al., 2010], a finding consonant with a recent family-based study [Smith et al., 2014], but not with other studies [Cho, Yoo, Park, Lee, & Kim., 2007; Guhathakurta et al., 2009; Hérault et al., 1996; Veenstra-Vanderweele et al., 2002]. Given the reported contribution of DNA methylation to altered *HTR2A* mRNA expression in some other mental health disorders [Abdomalekey et al., 2011; Falkenberg et al., 2011], it is tempting to assume that epigenetic mechanisms might contribute to *HTR2A* dysregulation in autism as well. Indeed, a subset of adult ASD patients from this study displayed diminished ADP-induced platelet aggregation (an indirect measure of the platelet HTR2A receptor activity/number) as compared to healthy controls [Hranilovic et al., 2009], supporting such hypothesis and indirectly pointing to biological relevance of the observed *HTR2A* promoter hypermethylation in autism. Our future intention is to employ HTR2A binding studies to further investigate interaction between genetic and epigenetic events in determining the function of HTR2A in autism.

Table 1. Control and Autistic Subjects Homozygous for rs6306 Major (C) Allele and Stratified According to rs6311 Genotype

	Control subjects (19–45 years)	Autistic subjects (19–45 years)	Autistic subjects (4–18 years)
GG genotype			
N (% of females)	8 (25)	16 (3)	13 (15)
Age [mean ± SD]	31.8 ± 9.8	27.1 ± 6.6[a,c]	12.0 ± 4.3[b,c]
AG genotype			
N (% of females)	12 (50)	15 (27)	16 (38)
Age [mean ± SD]	32.4 ± 8.6	28.4 ± 7.0[a,c]	10.4 ± 4.0[b,c]
AA genotype			
N (% of females)	11 (36)	10 (10)	5 (0)
Age [mean ± SD]	25.2 ± 6.8	28.4 ± 5.1[a,d]	11.6 ± 3.1[b,d]

[a] $P > 0.05$ vs. control subjects.
[b] $P < 0.001$ vs. control subject.
[c] Dunn's post hoc analysis after Kruskal–Wallis test.
[d] Bonferroni's post hoc test after one-way ANOVA.
N, number of subjects.

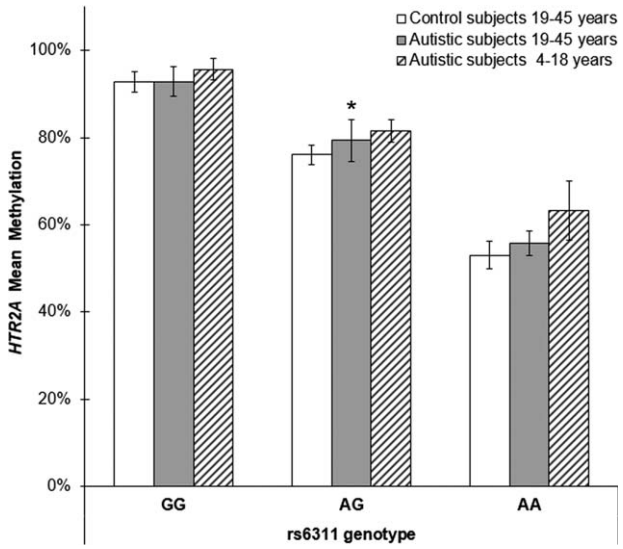

Figure 2. The *HTR2A* promoter methylation in control subjects (open bars), age-matched (19–45 years) autistic patients (filled bars) and autistic children (stripped bars) stratified according to rs6311 (−1438 A/G) genotype. Results are shown as means ± SD (for numerical data see Supporting Information Table 3). Only age-matched groups were compared. $*P < 0.05$ vs. control subjects (Dunn's post hoc analysis of Kruskal–Wallis test).

Study Limitations

Our sample had several limitations that could have hampered the obtained results. First, the lack of use of diagnostic instruments beyond DSM-IV criteria might have affected the composition of the patient sample. Second, a modest sample size remaining after the genotype grouping and age-matching considerably decreased the statistical power and might have represented a source of type I error. Third, most of our adult autistic subjects were medicated (see Supporting Information Table 1) what could have affected methylation in the ASD group. Neuroleptics and valproate were reported to decrease the *HTR2A* promoter methylation [Abdolmaleky et al., 2011]; escitalopram was also reported to decrease DNA methylation [Melas et al., 2012]. Since

our findings went in the opposite direction (i.e., increased DNA methylation in autistic subjects compared to the controls), and there were no differences in the methylation between medicated and nonmedicated autistic subjects in our study ($P = 0.939$, 0.546 and 0.602 for AA, AG and GG genotype, respectively), we consider the positive interference of medication as unlikely. Finally, our findings were obtained on blood leukocyte DNA and may not reflect *HTR2A* methylation status in the brain [Paquette & Marsit, 2014]. So far, similar [Ghadirivasfi et al., 2011] as well as different [De Luca et al., 2009] methylation profiles of the *HTR2A* promoter in the brain and peripheral cells have been reported.

Conclusions

We provide preliminary evidence for a higher DNA methylation within the *HTR2A* regulatory region in a portion of adult autistic subjects. This finding, obtained on the peripheral blood leukocytes, may contribute to understanding the dysregulation of the peripheral 5HT homeostasis in autism, but may also serve as a model for the events in the central 5HT compartment. Further studies in larger samples are needed to confirm our results and delineate the relationship between *HTR2A* methylation and its expression in autistic subjects.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Supplementary Table 1. Characteristics of studied subjects stratified according to diagnosis and age.

Supplementary Table 2. Correlation of methylation levels at loci −1522 (L1), −1439 (L2) and −1421 (L3) in subjects with a particular combination of rs6311 and rs6306 genotypes.

Supplementary Table 3. Mean methylation levels within the *HTR2A* promoter region in control and autistic subjects homozygous for rs6306 major allele (C) and stratified according to rs6311 genotype (means ± SD).