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Hyperserotonemia in Autism: The Potential Role of 5HT-related Gene Variants

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A B S T R A C T

Increased platelet serotonin level (PSL) has been consistently found in a portion of autistic patients. Suggested mechanisms for hyperserotonemia in autism have been increased synthesis of serotonin (5HT) by tryptophan hydroxylase (TPH), increased uptake into platelets through 5HT transporter (5HTt), diminished release from platelets through $5HT_{2A}$ receptor ($5HT_{2A}r$) and decreased metabolism by monoamine oxydase (MAOA). The allelic influence of genes, encoding the mentioned 5HT elements, on PSL was investigated in 63 autistic subjects. Our study shows that 5HTt-LPR and –1438AG $5HT_{2A}r$ genotypes did not significantly affect PSL. However, significantly higher PSLs were observed in subjects with »cc« genotype of a218c TPH and subjects with »4« genotype of uVNTR MAOA. In addition, when TPH-cc and MAOA-4 were combined as »high 5HT« genotypes, a correlative increase in PSL was observed with the increase in the number of »high 5HT« genotypes. These results suggest a possible synergistic effect of genes regulating 5HT synthesis/degradation in dysregulation of the peripheral 5HT homeostasis of autistic patients.

Key words: *autism, hyperserotonemia, serotonin transporter, tryptophan hydroxylase, monoamine oxydase A, $5HT_{2A}$ receptor*

Introduction

Autism is a neurodevelopmental syndrome characterized by disturbances in social interactions, language and communication, as well as by the presence of stereotyped behaviors and interests[1]. Prevalence of autism in general population is 3–6 per 1,000 with a high recurrence risk of 2–8% in siblings of the diseased, and up to 92% in monozygotic twins. This points to the strong genetic component of this disorder[2].

Several lines of evidence suggest that the alterations in serotonergic neurotransmitter system might represent one of the biological substrates of the disease. Serotonin (5-hydroxytryptamine, 5HT) has been shown to play an important role in brain development by regulating both, serotonergic outgrowth and maturation of target regions[3]. Pharmacological manipulation of 5HT transmission has been shown to influence some of the autistic symptoms[4–6], while positron emission tomography studies have demonstrated an altered brain 5HT synthesis capacity of autistic children[7,8]. Perhaps the most intriguing 5HT-related finding in autistic disorder is hyperserotonemia. For several decades elevated blood 5HT levels have been consistently found in about one third of the autistic patients[9], but the mechanism of the observed phenomenon has remained unclear. Increased synthesis of 5HT in the intestine[10], increased uptake of serotonin into platelets[11], diminished release from platelets[9], or decreased catabolism of 5HT[12] have been suggested as possible causes.

More than 99% of the whole blood serotonin is contained in platelets[9]. 5HT concentration in platelets, or

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the platelet serotonin level (PSL), is regulated by several elements that control either the rate of 5HT synthesis and metabolism (i.e. its concentration in blood plasma), or the rate of its accumulation into- and release from the platelets.

Tryptophan hydroxylase (TPH) is the rate limiting enzyme of 5HT synthesis. The peripheral isoform is encoded by the gene *Tph1* that contains a single nucleotide polymorphism (SNP), adenine to cytosine transition on the position 218 of the intron 7, termed a218c TPH[13].

Monoamine oxydase A (MAOA) catalyzes oxidative deamination of 5HT, an essential step in catabolism of monoamines. Its gene contains a 30 bp variable number of tandem repeats (VNTR) in the promoter (upstream) region, termed uVNTR MAOA[14]. Although alleles with 2, 3, 3.5, 4, 4.5, 5 and 6 repeats have been reported, variants with 3 and 4 repeats constitute more than 97% of the alleles in all reported control samples[15].

Serotonin transporter (5HTt) and serotonin receptor ($5HT_{2A}r$) are located on the platelet membrane. Serotonin transporter actively transports 5HT from blood plasma into the platelet. A 22 bp VNTR in the promoter region, termed 5HTt-LPR, results either in a 16-repeat long (L) allele or in a 14-repeat short (S) allele[16].

Activation of $5HT_{2A}$ receptor mediates the release of serotonin from the platelet in the process of platelet aggregation. Analysis of the promoter region of the gene revealed a SNP at the position –1438 that contains either adenine or guanine[17], termed –1438AG $5HT_{2A}r$.

With the exception of a218c TPH, expression studies suggested possible functional relevancies of the mentioned polymorphisms by showing allele-dependent differences in the promoter activities of the corresponding genes[14,16,18].

All of the mentioned 5HT-regulating peripheral proteins have their counterparts, encoded by the same genes, in the brain where they regulate 5HT neurotransmission. It might be assumed that the alterations in their expression could lead to hyperserotonemia in the periphery with simultaneous dysregulation of 5HT transmission in the brain, the two phenomena observed in autistic individuals. With this hypothesis in mind, we have studied the potential influence of the allelic variants of TPH, MAOA, 5HTt and $5HT_{2A}r$ genes on the platelet 5HT levels in a group of individuals affected with autism.

Subjects and Methods

Autistic patients were recruited from the Center for autism Zagreb, Croatia, after being examined by a psychiatrist and two psychologists. The group consisted of 63 subjects (47 males, 16 females, X±SD:20.2±9.0) diagnosed with autism spectrum disorders (54 with autism, 1 with Aspereger's syndrome, and 8 with PDD NOS), according to DSM-IV criteria. Severity of behavioral symptoms was measured using the Childhood Autism Rating Scale (CARS)[19]. Degree of mental retardation was assessed according to the standardized intelligence or developmental tests, depending on the apparent developmental level of each individual. Drug therapy included typical neuroleptics (7 patients), anticonvulsants (5 patients), and a combination of neuroleptics and anticonvulsants (26 patients, 18 with typical and 8 with atypical neuroleptics).

All subjects were of Croatian origin. After an informative talk, a written consent for inclusion in the study was obtained from the patients' parents. The study has been carried out in accord with the Declaration of Helsinki, and was approved by the Ethics Committee of the Medical Faculty of the University of Zagreb.

Blood sampling was performed between 9 and 11 a.m. Venous blood was collected into vacutainers containing ACD (for PSL determination) or EDTA (for genotyping) anticoagulants.

After a thorough mixing, blood was transferred to the 15 mL Falcon tubes and centrifuged at 1,050 × g for 2 minutes to obtain PRP. Separated PRP was aliquoted for an automated determination of platelet number and volume, and for measurements of 5HT concentration (in duplicates).

Platelet pellet, obtained by centrifugation of a diluted PRP sample (1 mL PRP + 3 mL saline) at 8,500 × g for 5 minutes, was sonicated in 1 mL of deionized water. Following deproteinisation with $ZnSO_4$/NaOH, 5HT content was measured by orthophtaldialdehyde-enhanced fluorometry at 345/485 nm, as previously reported[20]. Results were expressed as ng 5HT per μL of total platelet volume (calculated as the product of mean platelet volume and platelet count).

DNA was isolated from the whole blood using a DNA isolation kit for mammalian blood (Boehringer Manheim, Germany).

PCR for 5HTt-LPR polymorphism was performed in a total volume of 15 μL containing 1.5 mM $MgCl_2$, 0.8 mM dNTP mix (dATP, dCTP, dTTP, dGTP, 7-deaza-dGTP= 1:1:1:0.5:0.5), 0.5 mM primers (forward 5'-GGCGTTGCCGCTCTGAATGC-3', reverse 5'-GAGGGACTGAGCTGGACAACCAC-3'), 0.5 U Taq polymerase, and 200 ng template, with the addition of Q solution (Qiagen, Germany). Cycling conditions consisted of a 5-min denaturation at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 61 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products (528 bp allele »L« and 482 bp allele »S«) were separated on 2% agarose gel.

PCR for MAOA polymorphism was performed in a total volume of 15 μL containing 1.5 mM $MgCl_2$, 0.8 mM dNTP mix, 0.4 mM primers (forward 5'-ACAGCCTGACCGTGGAGAAG-3', reverse 5'-GAACGGACGCTCCATTCGGA-3'), 0.55 U Taq polymerase, and 200 ng template. Cycling conditions consisted of a 2-min denaturation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 61 °C and 40 s at 72 °C, and a final extension of 7 min at 72 °C. PCR products (306 bp allele »3« and 336 bp allele »4«) were separated on 3% agarose gel.

PCR for TPH polymorphism was performed in a total volume of 15 μL containing 2.0 mM $MgCl_2$, 0.8 mM dNTP mix, 0.5 mM primers (forward 5'-TTCAGATCCC-

TTCTATACCCCAGA-3', reverse 5'-GGACATGACCTAAGAGTTCAGGCA-3'), 0.55 U Taq polymerase, and 150 ng template. Cycling conditions consisted of a 3-min denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 40 s at 72 °C, and a final extension of 10 min at 72 °C. 5 μL of PCR products were digested with 5 U of FspBI (Fermentas, USA) in a total volume of 20 μL overnight. The obtained fragments (uncut allele »a« of 848 bp, and cut allele »c« of 597 bp and 251 bp) were separated on 1.2% agarose gel.

PCR for $5HT_{2A}r$ polymorphism was performed in a total volume of 15 μL containing 1.5 mM $MgCl_2$, 0.8 mM dNTP mix, 0.5 mM primers (forward 5'-AACCAACTTATTTCCTACCAC-3', reverse 5'-AAGCTGCAAGGTAGCAACAGC-3'), 0.55 U Taq polymerase, and 150 ng template. Cycling conditions consisted of a 5-min denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, and a final extension of 10 min at 72 °C. 5 μL of PCR products were digested with 5 U of MspI (Fermentas, USA) in a total volume of 20 μL overnight. The obtained fragments (uncut allele »A« of 468 bp, and cut allele »G« of 224 bp and 244 bp) were separated on 2% agarose gel.

Data were processed by the use of GraphPad InStat 3.01 software. Normality of distributions of the measured parameters was tested by Kolmogorov/Smirnov test, while the equality of SDs was tested by Bartlett's test. Mean PSL values were compared among different genotypes using unpaired t-test or one-way analysis of variance (ANOVA). Values of the parameters that were not normally distributed or that differed significantly in their SDs were log transformed prior to ANOVA. The level of significance was set to 0.05. Values in the text were expressed as means ± standard deviations (X±SD).

Results

The mean PSL value of the integral sample of autistic subjects was 74.0±33.8 ng/μL (the reported PSL values for Croatian[21] and white American[22] control subjects were 59.2±16.2 ng/μL and 68±17 ng/μL, respectively). No significant effects of gender ($F_{(1,62)}=0.13$, $p=0.72$), and therapy ($F_{(3,62)}=0.62$, $p=0.60$) on this parameter, nor its correlation with age ($r=-0.032$, $p=0.799$) was observed.

Genotypes of the four investigated polymorphisms, with the corresponding mean PSL values, are presented in Table 1.

SS and LL homozygotes of the 5HTt-LPR polymorphism displayed similar mean PSL values (69.8±24.9 ng/μL and 67.8±25.4 ng/μL, respectively), while the mean value of LS heterozygotes was somewhat higher (81.7 ±42.5 ng/μL). One subject carried 18-repeat extra-large (XL) allele (genotype XLL) and was not included in the statistical analysis. One-way ANOVA of the logarithmically transformed data did not show significant influence of this polymorphism on PSL values ($F_{(2,61)}=0.87$, $p=0.71$).

Similarly, polymorphism –1438 AG of the $5HT_{2A}r$ gene did not significantly affect platelet 5HT concentrations in the investigated sample ($F_{(2,62)}=0.90$, $p=0.15$), mean values for AA, AG and GG genotypes being 60.0 ±12.2 ng/μL, 81.5±42.9 ng/μL and 69.7±24.7 ng/μL, respectively.

Significant effect on the platelet serotonin level was found for the a218c polymorphism of the tryptophan hydroxylase gene ($F_{(2,62)}=3.37$, $p=0.04$). While the mean PSL values of individuals with aa and ac genotypes were almost identical (65.4±26.7 ng/μL and 65.4±22.8 ng/μL, respectively), the mean platelet serotonin concentration was about 25% higher in subjects homozygous for the allele c (87.2±42.1 ng/μL).

Since the gene for MAOA is located on chromosome X, only females can be homozygotes or heterozygotes for the investigated polymorphism, while the males are allele 3- or allele 4-hemizygotes. Mean PSL value of the three female 3/3 homozygotes were 41.0±21.7 ng/μL, of the four 3/4 heterozygotes were 67.3±36.0 ng/μL and of the seven 4/4 homozygotes were 87.4±25.6 ng/μL. One

TABLE 1
INFLUENCE OF THE GENOTYPES OF 5HT-RELATED GENES ON THE PLATELET SEROTONIN LEVEL (PSL) IN AUTISTIC SAMPLE

Polymorphism	Genotype	N	PSL (ng/μL)	Statistics
5HTt-LPR	SS	13	69.8±24.9	$F_{(2,61)}=0.87$
	LS	27	81.7±42.5	$p=0.71$
	LL	22	67.8±25.4	
–1438 AG $5HT_{2A}r$	AA	6	60.0±12.2	$F_{(2,62)}=0.90$
	AG	28	81.5±42.9	$p=0.15$
	GG	29	69.7±24.7	
a218c TPH	aa	13	65.4±26.7	$F_{(2,62)}=3.37$
	ac	25	65.4±22.8	$p=0.04$
	cc	25	87.2±42.1	
uVNTR MAOA	3/3 + 3/0	20	61.8±24.2	$t=2.43$, 56 df
	4/4 + 4/0	29	81.0±36.6	$p<0.02$

subject carried allele with 4.5 repeats (genotype 4/4.5) and was not included in the analysis. Mean values of the male 3/0 and 4/0 hemizygotes were 65.5±23.2 ng/μL and 79.8±39.3 ng/μL, respectively. Since MAOA seems to be expressed from only one of the two alleles in female cells[23], 3/3 homozygotes functionally correspond to the male 3/0 hemizygotes, while 4/4 homozygotes correspond to the male 4/0 hemizygotes. Heterozygotes are uninformative since they cannot be functionally grouped as expressing either of the alleles. When we compared the mean platelet 5HT concentrations of the subjects carrying 3/3 and 3/0 genotypes (61.8±24.2 ng/μL) to those of the subjects carrying 4/4 and 4/0 genotypes (81.0±36.6 ng/μL), we observed significantly higher value, of about 25%, in the latter group ($t=2.43$, 56 df, $p<0.02$).

We further investigated whether the polymorphisms a218c TPH and uVNTR MAOA, that influenced the level of platelet serotonin, had a combined effect on platelet 5HT concentrations. We considered TPH-cc and MAOA--4 as »high 5HT« genotypes, and grouped subjects according to the number of »high 5HT« genotypes (none – neither »cc« nor »4«, one – either »cc« or »4«, and two – both »cc« and »4«). We have observed a significant difference in the mean 5HT levels among the three groups ($F_{(2,57)}=6.175$, $p<0.004$, one-way ANOVA), with a clear dose-effect. There was an increase in the mean 5HT levels with the increase in the number of »high 5HT« genotypes ($F_{(1,57)}=12.08$, $p=0.001$, post-ANOVA test for linear trend), mean values being 57.8±25.8 ng/μL, 69.4±20.1 ng/μL, and 94.2±46.6 ng/μL, respectively (Figure 1).

Discussion

In this study, we have investigated the influence of 5HT-related gene polymorphisms on the platelet serotonin level in an ethnically homogenous group of 63 subjects rather severely affected with autism (mean CARS score of 42.2±8.4). Relatively high percentage of medicated subjects (60%) and a wide range of age (4-39 years) might have affected the reliability of the PSL measurements and represent the limitations of our sample. However, regarding the first, subjects receiving SSRI which directly influence platelet 5HT concentrations were not included in the study. Other medication not directly acting on 5HT system did not seem to have any effect on the measured PSL values, what is in line with the observations reported by Mulder et al.[24] and with our observations from the previous study[21]. Regarding the second, although some authors suggested the effect of puberty on platelet 5HT levels of autistic individuals[22], the mean PSL values of 16 pre-pubertal children (69.1±19.8 ng/μL) and 47 adults (75.7±37.4 ng/μL) in our sample did not significantly differ ($t=0.166$, $p=0.869$, 48 df, Welch's corrected t-test) what allowed for the joint analysis of the data.

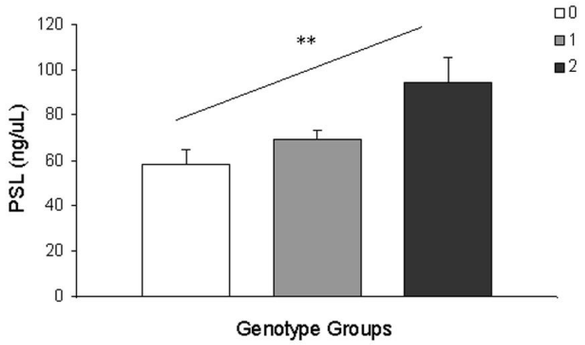

*Fig. 1. The effect of the number of »high 5HT« genotypes on the platelet serotonin level (PSL) in autistic subjects. Genotype »cc« of a218c TPH and genotype »4« of uVNTR MAOA were considered as »high 5HT« genotypes. 0 – no »high 5HT« genotypes (subjects containing neither »cc«, nor »4«, N=13), 1 – »high 5HT« genotype at one locus (subjects containing either »cc«, or »4«, N=27), 2 – »high 5HT« genotypes at both loci (subjects containing both, »cc« and »4«, N=18). Results are presented as X±SEM, **p=0.001, post--ANOVA test for linear trend).*

Significant correlations between the platelet serotonin level and the platelet serotonin uptake have been reported in a group of autistic children[25] and adults[26]. However, we have not found a significant effect of the 5HTt-LPR genotypes on the platelet 5HT concentrations, what is in line with the previously reported findings on autistic subjects[25,27–29]. Therefore, it could be assumed that even if the 5HT transporter gene is involved in development of hyperserotonemia in autistic individuals, the mechanism of its action does not likely include the mentioned polymorphism. Indeed, 5HTt-LPR represents one of the most studied polymorphisms in autism, but the equal representation of the studies associating allele S, allele L, or neither of the alleles with autistic symptoms[30] makes its role in autism far from understood.

Receptor binding studies suggested a decreased expression of $5HT_{2A}$ receptor on the platelets of autistic subjects[31] and their hyperserotonemic first-degree relatives[26], what made it an interesting candidate gene for the association studies in autism. However, the only study of association of the –1483AG $5HT_{2A}$r polymorphism with autism, conducted so far, did not reveal preferential transmission of either of the alleles from heterozygous parents to the affected offspring[32]. In our study, platelet 5HT concentrations did not differ among individuals carrying different –1483AG $5HT_{2A}$r genotypes, suggesting the lack of the involvement of this polymorphism in the development of hyperserotonemia.

The enzyme TPH1 regulates the rate of 5HT synthesis in the periphery, but is also shown to be expressed in the fetal brain, possibly regulating the development of 5HT neurons[33]. So far, only one study testing the association of a218c TPH polymorphism with autistic disorder has been reported, with negative results[34]. We have found a significant effect of the mentioned polymorphism on the 5HT concentrations in platelets of autistic individuals, subjects with the two »c« alleles having higher PSL values than the subjects with the other two genotypes. Although functional variants affecting TPH expression have not yet been identified, our finding indicates that a218c SNP, or some other nearby polymorphism, might affect the ex-

pression of *Tph1* gene, and implicates the role of TPH1 in the development of hyperserotonemia.

Due to its role in metabolizing serotonin and catecholamines, as well as to its location on the chromosome X (autism being a predominantly male disorder), MAOA gene has also attracted interest as a potential candidate gene in autism. Although the two reported family-based association studies did not find preferential transmission of either of the uVNTR MAOA alleles, both studies revealed a significant effect of the mentioned polymorphism on IQ of the autistic probands[35,36]. We have found significantly higher mean PSL values in a group of subjects carrying »genotype 4« in comparison to those carrying »genotype 3«, what indicates the involvement of this functional polymorphism in the development of hyperserotonemia.

Finally, when TPH-cc and MAOA-4 were considered as »high 5HT« genotypes, a significant increase in the mean 5HT concentrations was observed with the increase in the number of »high 5HT« genotypes. The mean PSL value of the group having both, »cc« and »4« genotypes was considerably increased and in the range of previously determined hyperserotonemia[21]. The observed effect would remain significant even if we would correct the level of significance to 0.01 (due to the multiple testing of four genotypes and one combination) what points to the possible synergistic effect of the two genes in regulation of the peripheral 5HT levels.

Since the discovery of hyperserotonemia in autism, there has been a debate over what causes the elevated 5HT levels in blood – increased concentrations of plasma free 5HT, increased accumulation of 5HT in platelets, or a combination of both[37,38]. In our sample, we have observed a weak individual and a stronger combined influence of a218c TPH and uVNTR MAOA genotypes on 5HT levels in platelets of autistic subjects. Although the functionalities of the studied polymorphisms remain to be clarified, the obtained results indicate that the genes regulating 5HT synthesis/degradation rate (TPH and MAOA) rather than the genes regulating 5HT transfer in and out of platelets (5HTt and $5HT_{2A}r$) might be involved in dysregulation of the peripheral 5HT homeostasis, and suggest their potential role in development of autistic disorder. Before drawing any conclusions, we have to check the influence of the investigated genotypes on PSL in a control sample and test the association of the corresponding genes with autistic disorder, what represents the objects of our future studies.

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HIPERSEROTONINEMIJA U AUTIZMU: MOGUĆA ULOGA INAČICA GENA VEZANIH UZ 5HT SUSTAV

S A Ž E T A K

U dijelu autističnih pacijenata uočena je povišena razina trombocitnog serotonina (engl. *platelet serotonin level*, PSL). Kao mogući uzroci spominju se pojačana sinteza serotonina (5HT) pomoću enzima triptofan-hidroksilaze (TPH), pojačani unos serotonina u trombocite putem 5HT prijenosnika (5HTt), smanjeno otpuštanje serotonina iz trombocita preko $5HT_{2A}$ receptora ($5HT_{2A}$r) i smanjena razgradnja serotonina pomoću enzima monoamin-oksidaze (MAOA). U ovom radu istražen je utjecaj alela gena, koji kodiraju spomenute elemente 5HT sustava, na PSL u 63 autistične osobe. Dok genotipovi 5HTt-LPR i -1438AG $5HT_{2A}$r nisu značajno utjecali na PSL, osobe s genotipom »cc« polimorfizma a218c TPH i osobe s genotipom »4« polimorfizma uVNTR MAOA imale su značajno povišeni PSL. Kada smo genotipove TPH-cc i MAOA-4 označili kao »genotipove s visokim 5HT«, uočili smo značajan porast PSL s porastom broja »genotipova s visokim 5HT«. To upućuje na mogući sinergistički učinak gena koji reguliraju sintezu/degradaciju serotonina na poremećaj regulacije periferne 5HT-homeostaze u autističnih osoba.