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Platelet and intestinal 5-HT_{2A} receptor mRNA in autistic spectrum disorders – results of a pilot study

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The etiology and pathogenesis of autistic spectrum disorders (ASD) are still unknown. Platelet hyperserotonemia has been detected in 25-60% of autistic children. Higher incidence of gastrointestinal problems in people with autism is observed. The aim was compare the expression of platelet 5-HT_{2A}r mRNA in autistic and non autistic groups. In a subgroup of patients with gastrointestinal problems an upper gastrointestinal tract endoscopy was performed and additionally the expression of 5-HT_{2A} receptor mRNA in the duodenum was assessed. The examination was conducted in 79 children - 51 with ASD and 28 without autistic traits. Statistically significant differences between the study and control groups were proven in gastrointestinal problems. The analyses reveal a significantly higher level of 5-HT_{2A}r mRNA in platelets of the study group patients, which could suggest serotonin system dysregulation.

Key words: infantile autism, autistic spectrum disorders, serotonin, serotonin receptor, 5-HT_{2A}r mRNA, gastrointestinal problems

Infantile autism (IA) and atypical autism (AA) are referred to as autistic spectrum disorders (ASDs). The incidence is estimated at 7 per 10 000 children, and is several times higher (20 per 10 000) for atypical autism (Williams et al. 2006).

ASDs do not have any etiological factor in common. In ASDs abnormal levels of neurotransmitters have been observed, including gamma-aminobutyric acid (Dhossche 2002), glutamine acid (Carlsson 1998, Friedman et al. 2003), endorphins (Wakefield et al. 2002) and catecholamine's (Perry et al. 2001). The concept of disorders in serotonergic transmission is the most prevalent; platelet hyperserotonemia has been detected in 25-60% of autistic children (Singh et al. 1997, Chugani et al. 1999, Leboyer et al. 1999, Croonenberghs et al. 2000, Anderson et al. 2002, Betancur et al. 2002, Kahne et al. 2002, Persico et al. 2002, Janusonis 2005a, Janusonis et al 2006, Boylan et al. 2007).

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Although serotonin (5 – hydroxytryptamine; 5-HT) is a neurotransmitter of the nervous system (central nervous system - CNS, peripheral nervous system - PNS, enteric nervous system - ENS), it can also be found extraneuronally in blood platelets and the intestinal mucous membrane cells. 5-HT is synthesized in neurons and enterochromaffin cells of the intestinal mucosa (95% of all 5-HT in the body is present in the gastrointestinal tract).

Seven main groups of 5-HT receptors have been distinguished; 16 subtypes of serotonin receptors have been identified up to the present. In neuropsychiatry dysregulation of the 5-HT_{2A} receptor ($5HT_{2A}$r; 13q14-q21) is observed in obsessive compulsive disorders, mood disorders, eating disorders, and drug dependence syndrome.

It has been found that the molecular structure of neuronal and platelet $5HT_{2A}$r mRNA is identical, and the pharmacological properties of the $5HT_{2A}$r in platelets and neurons are similar. The structure of serotonin transporter (SERT) proteins in platelet and in neurons is identical, and their kinetic properties

are very similar. Thus the platelets do share some features with 5-HT synaptosomes (Da Prada et al. 1988, Pletscher 1988, Cook et al. 1994, Berk et al. 2003, Janusonis 2005a, Rausch et al. 2005, Carneiro et al. 2006, Uebelhack et al. 2006). More than that, the 5-HT present in the GI tract is taken into the intestinal epithelial cells by SERT identical to those present in the neurons of the ENS and CNS. Additionally $5HT_{2A}$rs identical with the neural $5HT_{2A}$rs are found on smooth muscle cells and cells of the gastrointestinal (GI) mucous membrane, including enterochromaffin cells, where they are likely to function as serotonergic autoregulators (Spiller 2002, Bellini et al. 2003).

Over the past few years attention has been paid to the higher incidence of GI problems in ASD patients, compared to the population of healthy children (Horvath et al. 1999, Wakefield et al. 2000, Torrente et al. 2004, Parracho et al. 2005, Valicenti-McDermott et al. 2006).

To the knowledge of the authors, the profile of platelet and intestinal $5HT_{2A}$r mRNA has not yet been studied. The aim of authors was to compare the expression of platelet and intestinal 5-HT_{2A}r mRNA in autistic and non autistic groups.

The children were patients of the Department of Pediatrics and Child Neurology at the Silesian Medical University. The analysis included a total of 79 people, aged between 36 months and 12 years. Study group - 51 patients; males n=39, females n=12; aged between 36 months and 13 years; mean age 8 years 1 month; IA: 25 patients; AA: 26 patients. Control group – patients without autistic traits - 28 persons; males n=20; females n=8; aged between 36 months and 12 years; mean age 7 years 9 months. The study and control groups were homogenous in terms of age (Z=1.87; P=0.8213, Mann-Whitney U test) and sex (χ^2=0.24; P=0.6220, Pearson's *Chi*-square test). The data were obtained from a specially designed questionnaire filled in with parents during the first appointment with a doctor. Exclusion criteria included taking medications influencing 5-HT level (during 30 days prior to the trial), active epilepsy, presence of an organic change within the abdominal and/or pelvic cavity, and use of a gluten-free diet over the last 6 months. The study had two stages. The first stage entailed pediatric examination; anthropometric measurements and determination of the level of $5HT_{2A}$r mRNA in the platelets in relation to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) constitutive gene. Blood samples were obtained during a routine check–up or during another laboratory examination at the hospital. During the sample collection 3 ml of blood was taken. Each test was performed in spring, between 7:30 and 8:30 a.m. At the second stage, children with digestive problems had GI diagnostic tests, including endoscopy of the upper GI tract with duodenal mucosal biopsies, histological tests and marking of intestinal $5HT_{2A}$r mRNA in relation to the GAPDH constitutive gene.

The method of total RNA isolation from platelet/tissues according to Chomczynski and Sacchi, 1987 modified by the use of TRIzol reagent by Invitrogen: platelet-rich plasma was obtained from the patients' blood collected into tubes containing 3.8% sodium citrate solution in the proportion of 9:1. Tissue samples were homogenized in TRIzol reagent (1 ml/100 mg tissue) using a DIAX 900 homogenizer by Heidolph, with blades adjusted to small volume samples. The homogenate was then incubated for 5 min. at room temperature. Then, it was supplemented with 200 µm of chloroform (Molecular Biology Sigma), vigorously shaken, incubated for 3 min. at 30°C, and finally centrifuged at 15 000g for 15 min. at 4°C. Following the centrifugation, the mixture separated into 3 phases: a lower red phenol-chloroform phase, an interphase, and an upper colorless aqueous phase. The upper aqueous phase was transferred to a fresh Eppendorf tube, 500 µm of isopropyl alcohol was added (Molecular Biology Sigma), and incubated for 10 min. at 30ºC. The obtained mixture was centrifuged at 15 000 g for 10 min. at 4ºC and RNA precipitated as a pallet on the bottom of the tube. The upper layer was removed and the pellet was washed with 1 ml of 75% ethanol (DEPC water). All of that was centrifuged at 7 500 g for 5 min. at 4ºC. The tube containing the RNA was lightly drained and supplied with 30 µl of Molecular Biology Grade Water deprived of RNAase. The water-dissolved RNA was incubated for 10 min. at 60°C. The thus isolated RNA samples were stored at the temperature of -70°C before using for RT-PCR. The content and purity of RNA isolated from the tissues were measured using the spectrophotometric method at λ=260 nm and λ=280 nm. The measurements were taken with the use of a BioFotometr by Eppendorf, and disposable cuvettes Uvette.

RT-PCR reaction according to E.H.Cook et al. 1994, with our own modification. The primer selection used for an outer and nested reaction (Table I).

Table I

Primers used in outer and nested RT – PCR reaction.

	Sense Primers	Antisense Primers	Product length (bp)
Name	5’ 3’	5’ 3’	
GAPDH constitutive gene	**11** CgT ATT ggg CgC CTg gTC ACC	**22** ggg ATg ATg TTC Tgg AgA gCC C	**587**
P1 outer	**P1** TAA CCT gTT AgT CCT TCT ACA CCT C	**P22** TTC Cag ATA ggT gAA AAC TTg CTC	**1526**
D1 nested	**D1** TgC TAC AAg TTC Tgg CTT AgA CAT gg	**D22** ATg gCg CAg Agg TgC ATg ATg	**528**
D3 nested	**D3** TCT ggA TTT ACC Tgg ACg TgC	**D44** gCT TCT TTC Tgg AgT gAC TTg AT	**350**
P3 nested	**P3** ggC ATT TCT gAA AAT CAT Tg	**D55** TgC CTT CCA Cag TTg CCA Cgg C	**876**

The degrees of GAPDH and 5-HT_{2A} gene expression were evaluated by the reverse transcription polymerase chain reaction **(**RT-PCR) reaction. RNA solution samples of 100 ng / 5 µl were used for amplification. After initially incubating 200 ng/5 µl of the total RNA and 10 µM of P22 antisense primer for 5 minutes at 70°C, the mixture was cooled to 4°C. Then it was supplemented with 10 mM dNTPs/1 µl, 1 mM MgSO4/1 µl and 5U/1 µl of avian myeloblastosis virus reverse transcriptase, in a buffer from the Promega ACCESS set. The first strand reaction (RT) was carried out in an Eppendorf thermocycler for 45 minutes at 45°C.

Outer PCR reaction. The RT product was added to 10 µM of P1 antisense primer, 10 mM dNTPs/ 1µl, 2mM MgSO4/2 µl and 5U of *Thermus flavus* DNA polymerase per 40 µl of PCR mixture, in a buffer from the Promega ACCESS set. The outer PCR reaction profile, Eppendorf thermocycler: initial denaturation at 94°C for 2 minutes; annealing at 94°C for 30 seconds, at 58°C for 1 minute, at 68°C for 2 minutes, in a series of 30 cycles; final elongation at 68°C for 7 minutes. The outer PCR 1,526 byproduct was not visible on agarose gel for short DNA fragments.

PCR reaction was set with nested primers and 5 µl of the PCR product was used. Nested PCR reaction profile, Eppendorf thermocycler: initial denaturation at 94°C for 2 minutes; annealing at 94°C for 30 seconds, at 55°C for 1 minute, at 68°C for 2 minutes, in a series of 30 cycles; final elongation at 68°C for 7 minutes.

The most prominent products were obtained with the use of D1-D22 primer pair. To identify the products, electrophoresis was applied on 2% ethidium bromide-stained LMP (low melting point) agarose for short DNA fragments, produced by Promega, with the presence of the pUC19/MspI DNA marker: 501, 489, 404, 331, 242, 190, 147, 11 bp. The GAPDH constitutive gene products were obtained by means of an outer RT-PCR reaction. Densitometric measurements of the bands resulting from electrophoretic separation of nested RT-PCR products were taken using LabWorks software by UVP, Laboratory Products. The degree of 5-HT_{2A} gene expression in relation to the constitutive gene was evaluated statistically.

The database of clinical material was constructed by means of the Excel 2000 spreadsheet by Microsoft, and was then implemented in the Statistica 7.1 software by StatSoft. In statistical analysis, a p-value$\leq$0.05 was considered significant. At the first stage we determined the basic characteristics of descriptive statistics for every quantitative parameter. Next the frequency of every qualitative variable in particular groups and subgroups were assessed. At the second stage Student’s *t*-test, Mann-Whitney *U* test, *Chi*-square analysis and Spearman’s rank correlation test were performed.

Table II

Study group characteristics.

	Autistic group	Control group	χ^2 test ; *P*
Abdominal pain	56.9% ; 29/51	14.3% ; 4/28	χ^2=13.47 ; *P*<0.001
Sudden irritability episodes	66.7% ; 34/51	14.3% ; 4/28	χ^2= 19.87 ; *P*<0.001
Nighttime awakening	64.7% ; 33/51	25.0% ; 7/28	χ^2=11.40 ; *P*<0.001
Constipation	54.9% ; 28/51	3.6% ; 1/28	χ^2= 20.50 ; *P*<0.001
Diarrhea	41.2 % ; 21/51	7.1% ; 2/28	χ^2=10.15 ; *P*=0.0014
Eating rituals	58.8% ; 30/51	0.0% ; 0/28	χ^2=26.55 ; *P*<0.001

Autistic patients displayed GI problems more frequently than their non-autistic peers. The most important of them included abdominal pain, sudden irritability episodes, nighttime awakening, constipation, and diarrhea (*P*<0.001) (Table II). Endoscopy of the upper GI tract was needed in 41% (n=21/51) of the ASD patients (vs. 3.5% in the control group). In the course of endoscopic examinations, the patients had duodenal mucosal biopsies taken. In ASD patients the histological picture was abnormal in 18 of 21 (87.7%). In most cases chronic duodenitis was diagnosed (18/21; 85.7%).

In each patient, platelet mRNA was labeled for the $5HT_{2A}$r and GAPDH. The analyses reveal a significantly higher level of $5HT_{2A}$r mRNA in platelets of the study group patients (*Z*=6,61; *P*<0.001) (Table III). In all the patients who had the upper GI tract endoscopy performed and mucosal biopsies taken from the descending duodenum, the levels of 5-HT_{2A}r mRNA and GAPDH mRNA were identified. Twenty-one outcomes were obtained: 18 in the study group (18/21; 85.7%), and 3 in the control group (3/3; 100%). In view of the small number of patients in the control group, the sample is not representative as a control sample and it was not possible to analyse the results statistically. Within the study group, dependencies between platelet $5HT_{2A}$r mRNA and intestinal mucosal $5HT_{2A}$r mRNA were analyzed. The Spearman rank correlation test applied in the analysis revealed no correlation between the platelet and mucosal $5HT_{2A}$r mRNA (*R*=0.45; *P*=0.057). The analyses were supplemented by comparing the patients with IA to those with AA within the study group. The difference between the groups was not significant.

Abnormal 5-HT level, as with other neurotransmitters, may result from problems in its synthesis, metabolism, release, transport, or receptors (hypo- or hypersensitivity). Tryptophan bioaccessibility, SERT dysfunction (SERT promoter polymorphism and post-translational modifications), monoamine oxidase A were analysed in ASD patients by a number of scientific groups (Croonenberghs et al 2000, Persico et al 2002, Cohen et al 2003). Some research concentrates on the analysis of $5HT_2$r in ASD patients. In the research carried out by McBride and coworkers (1989) a reduced number of 5 HT_2 binding sites were reported in the platelets of young autistic adults. Cook and colleagues (1993) discovered a negative correlation between the number of 5- HT_{2A} binding sites and the platelet 5-HT level in first degree relatives of patients with autism. Hranilovic and coauthors (2009) measured the activity SERT, and MAO B and indirectly studied the activity of $5HT_{2A}$r. Their results suggest a possibility of upregulation of monoaminergic synthesis/degradation and downregulation of $5HT_{2A}$r in autistic subjects. Our analyses reveal a significantly higher level of $5HT_{2A}$r mRNA in the platelets of the study group patients. Perhaps the discrepancy between the presented findings and ours is due to the selection of the analyzed groups. Our colleagues examined adult patients and as we know during development the function of receptor / neurotransmitter systems changes together with the types of serotonin recep-

Table III

Analysis of the serotonin profile in the study and control groups. The elements tested: platelet 5-HT_{2A} receptor mRNA ($5HT_{2A}$ mRNA plt), platelet index of GAPDH mRNA / platelet 5-HT_{2A} receptor mRNA (plt index), GAPDH mRNA (GAPDH plt), GAPDH mRNA (GAPDH mRNA gut), intestinal $5HT_{2A}$ receptor mRNA ($5HT_{2A}$ gut),and intestinal index of intestinal GAPDH mRNA / intestinal 5-HT_{2A} receptor mRNA (gut index);

	Autistic group		Control group		*Z* (U Mann-Whitney test); *P*
	N	mean ± SEM	N	mean ± SEM	
$5HT_{2A}$ mRNA plt	51	1343.4 ± 24.0	28	1062.8 ± 20.3	*Z*=6.61 ; *P*<0.001
GAPDH mRNA plt	51	912.0 ± 14.8	28	912.3 ± 16.0	*Z*=0.04 ; *P*=0.9673
$5HT_{2A}$ mRNA / GAPDH mRNA plt	51	1.47 ± 0.01	28	1.16 ± 0.01	*Z*=6.98 ; *P*<0.001
$5HT_{2A}$ mRNA gut	18	1069.4 ± 20.3	3	1255.5 ± 52.9	–
GAPDH mRNA gut	18	1600.8 ± 16.0	3	1809.7 ± 46.6	–
$5HT_{2A}$ mRNA / GAPDH mRNA gut	18	0.67 ± 0.02	3	0.69 ± 0.03	–

tors, their activity, number and location. The level of serotonin also changes with a general decreasing tendency - from physiological hyperserotoninemia during fetal development, through high levels of serotonin during further development and a stable low level of serotonin after reaching puberty (Chugani et al. 1999, Croonenberghs et al. 2000). Also as the body ages the number of $5HT_{2A}$r decreases for example by 70% at the age of 50 (Chugani et al. 1999, Sheline et al. 2002, Volgin et al. 2003). Therefore comparison of our and the above mentioned studies seems difficult. Murphy and others (2006) on the basis of the results of the SPECT study described decreased cortical $5HT_{2A}$r binding in Asperger syndrome. Canadian scientists (Goldberg et al. 2009) assessed serotonergic neurotransmission in the parents of ASD children, concluding on the basis of the PET results a significant decrease in the cortical 5-HT_2 binding potentials in the analysed group with concurrent negative corelation with the level of serotonin in blood platelets.

We are aware of the limitations of our study. Most patients with ASD and GI complaints were diagnosed with duodenitis. This is significant as we may not exclude the fact that the bowels, both in the aspect of increased intestinal synthesis of serotonin (Cronenberghs et al. 2005) and disturbed process of release of 5-HT from the enterochromaffine cells, (Janusonis 2005b) may be a significant factor to disturbed serotonin homeostasis. The details of the impact of inflammation on intestinal production/release of serotonin remain unknown (Bertrand et al. 2010). Moreover, despite biochemical and molecular homogeneity, post-translational modifications of $5HT_{2A}$r are possible (Cook et al. 1994).

We realize that our findings, including a statistically significant increase in the $5HT_{2A}$r mRNA level in ASDs children compared to their age, matched controls and a tendency for direct proportionality between $5HT_{2A}$r mRNA in platelets in the intestinal mucosa accounts for only a small portion of broad serotonergic dependencies. Close correlations between the levels of $5HT_{2A}$r mRNA in the CNS, platelets and the GI system

remain unknown. However, bearing in mind that there is scientific evidence of functional similarity between the neuronal and platelet serotonergic systems, we think the interrelations occurring in these systems are worth further analysis. Therefore, we would like to raise the question of the relationship between platelet hyperserotonemia, ASD symptoms and GI disorders and to carry out the second stage of the project which will enable us to consider either measurements of receptor protein level or receptor activity in the platelets and intestinum.

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