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HTR1B and *HTR2C* in autism spectrum disorders in Brazilian families

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ABSTRACT

Autism spectrum disorders (ASD) is a group of behaviorally defined neurodevelopmental disabilities characterized by multiple genetic etiologies and a complex presentation. Several studies suggest the involvement of the serotonin system in the development of ASD, but only few have investigated serotonin receptors. We have performed a case-control and a family-based study with 9 polymorphisms mapped to two serotonin receptor genes (*HTR1B* and *HTR2C*) in 252 Brazilian male ASD patients of European ancestry. These analyses showed evidence of undertransmission of the HTR1B haplotypes containing alleles –161G and –261A at *HTR1B* gene to ASD ($P=0.003$), but no involvement of *HTR2C* to the predisposition to this disease. Considering the relatively low level of statistical significance and the power of our sample, further studies are required to confirm the association of these serotonin-related genes and ASD.

1. Introduction

Autism is a complex neurodevelopmental disorder characterized by qualitative impairments in communication, social interaction, and restricted and repetitive patterns of interests or behaviors (American Psychological Association, 1994). Onset is generally before 3 years of age and results in lifelong disabilities for affected children. The prevalence is 1–2:1000 for narrow diagnosis of autism and 6:1000 for autism spectrum disorder (ASD), which includes Asperger syndrome and pervasive developmental disorders not otherwise specified (PDDNOS) (American Psychological Association, 1994; Chakrabarti and Fombonne, 2005). Autism is also four times more frequent in males than in females (Folstein and Rosen-Sheidley, 2001). Under its notable heterogeneity, abnormalities in the serotonin system have been suggested to play a

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Table 1 – Case-control analysis of HTR2C and HTR1B markers

Gene	Markers	Allele	Case % (*n*)	Control % (*n*)	Genotypes cases			Genotypes controls			χ^2 (*df*)	*p*-value
HTR1B	−261A>T	−261T	59%	58%	TT	TG	GG	TT	TG	GG		
		−261G	41%	42%	78	110	37	62	96	31	0.18	0.0914
	−182INS/DEL-181	−181delCC	97%	99%	Del	Del/ Ins		Del	Del/ Ins			
		−182insCC	3%	1%	218	14		186	4		3.95	0.047
	−161T>G	c.−161A	69%	70%	AA	AT	TT	AA	AT	TT		
		c.−161T	31%	30%	91	90	15	92	83	14	0.196	0.907
	c.861G>C	c.861G	75%	76%	GG	GC	CC	GG	GC	CC		
		c.861C	25%	24%	115	75	14	119	88	6	4.11	0.128
HTR2C	c.649+16895GA	c.649+16895G	86%	82%	G	A		G	A			
		c.649+16895A	14%	18%	208	34		203	43		1.08	0.322
	c.763+30G>A	c.763+30G	95%	98%	G	A		G	A			
		c.763+30A	5%	2%	232	11		244	4		3.519	0.06
	c.796G>C	c.796G	86%	82%	G	C		G	C			
		c.796C	14%	18%	208	34		199	42		1.039	0.308
	c.1078−31G>A	c.1078−31A	92%	90%	A	G		A	G			
		c.1078−31G	8%	10%	209	17		212	24		1.001	0.317

role in the development of ASD based on the recurrent observation of platelet hyperserotonemia in autistic children (Schain and Freedman, 1961; Janusonis et al., 2006; Cheh et al., 2006; Altamura et al., 2007; Boylan et al., 2007). Besides, drug treatment studies have suggested that selective serotonin reuptake inhibitor drugs can effectively reduce some symptoms such as repetitive behavior, aggression and problems with language use in individuals with ASD (McDougle et al., 2006). Therefore, genes encoding proteins involved in the serotonergic system have been considered as plausible candidates for ASD (Nabi et al., 2004; Coon et al., 2005; Weiss et al., 2006; Cho et al., 2007; Coutinho et al., 2007; Cross et al., 2008). Although still controversial, some studies have showed that polymorphisms of the serotonin transporter *SLC6A4* and *ITGB3* genes predispose to autism (Conroy et al., 2004; Coutinho et al., 2004; McCauley et al., 2004; Stone et al., 2004; Cantor et al., 2005; Weiss et al., 2006; Guhathakurta et al., 2008). The epistatic effect between the serotonin transporter genes *SLC6A4* and *ITGB3* in the determination of higher platelet serotonin levels have further reinforced the role of these genes in autism etiology (Coutinho et al., 2007; Carneiro et al., 2008). In contrast, only few studies have investigated serotonin receptors (Veenstra-VanderWeele et al., 2002; Janusonis, 2005; Janusonis et al. 2006; Coutinho et al., 2007; Cho et al., 2007) and so far there are no reports of *HTR1B* or *HTR2C* genes and autism.

The serotonin receptor 5-HT_{1B}, encoded by *HTR1B* gene, mapped at 6q13, plays an inhibitory role on serotonin neurotransmission by blocking the firing of the neurons and vesicle release (Sodhi and Sanders-Bush, 2004). The 5-HT_{2C} receptor, encoded by *HTR2C*, mapped at Xq24, is one of the main targets for respiridone and olanzapine, which are common drugs used in autism treatment (McDougle et al., 2006). These genes are expressed mainly in brain regions implicated in autistic phenotype as hippocampus, amygdala, and Purkinje cells (Boschert et al., 1994; Amaral et al., 2008). Moreover, polymorphic variations at *HTR1B* and *HTR2C* are associated with attention deficits, hyperactivity and

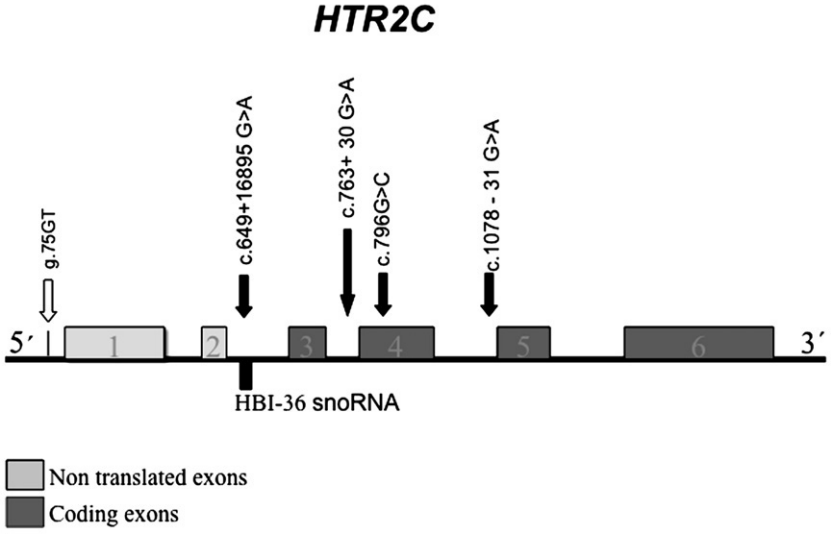

Fig. 1 – Schematic location of polymorphisms tested in the HTR2C gene. Precise sizes of introns and exons were not represented.

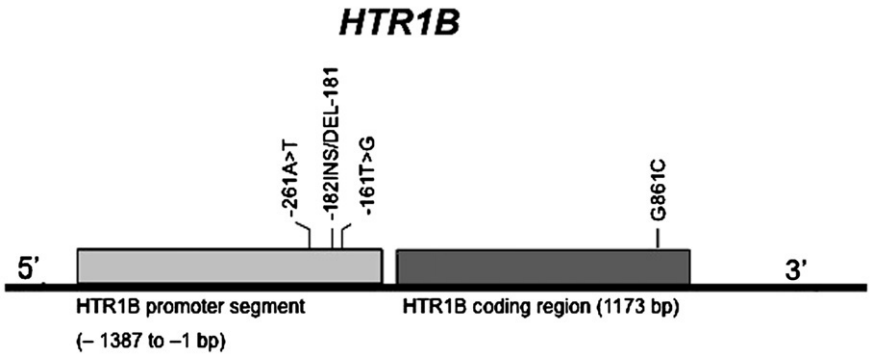

Fig. 2 – Schematic location of polymorphisms tested in the HTR1B gene. Precise sizes of promoter region and exon were not represented.

obsessive compulsive behaviour, which are disorders that include some symptoms that can also be seen in individuals with ASD (Hawi et al., 2002; Quist et al., 2003; Li et al., 2006; Massat et al., 2007).

Given the evidences of the *HTR1B* and *HTR2C* involvement in ASD, the present study sought to asses the association among 8 polymorphisms in these genes and Brazilian male ASD patients, using case-control and family based approaches. We did not find any evidence that SNPs at *HTR2C* play a role in the susceptibility of ASD. However, our results suggest a role of *HTR1B* in the predisposition of ASD, which warrents further investigated in a larger sample.

2. Results

Initially, we carried out a case-control study among 7 markers mapped in two genes coding for serotonin receptors, *HTR1B* and *HTR2C*, and ASD. Genotypes of all markers tested were in Hardy–Weinberg equilibrium ($P>0.05$) both in cases and controls. We observed that the alleles –182INSCC/*HTR1B* and c.763+30A/*HTR2C* were more frequent in ASD, but with borderline level of significance (Table 1; Figs. 1 and 2, Supplementary material). We observed a total of 9 different *HTR1B* haplotypes with similar distribution between cases and controls (*HTR1B*, $\chi^2=4.82$ *df* 8, global $P=0.77$). Four most prevalent *HRT2C* haplotypes were observed, and their distribution differed between cases and controls with a low level of significance (*HTR2C*, $\chi^2=9.757$, *df* 4; global $P=0.04$; data available on request).

We also conducted a family-based study to test if these SNPs represent risk-factors for ASD. For this analysis, we also included the data of the microssatelite marker g.75GT(16_21) at *HTR2C*. The family-based study of the individual markers at *HTR2C* and *HTR1B* confirm that there is no association between these markers and ASD ($P>0.05$). Using the same approach, we also did not find evidence of association between *HTR2C* haplotypes and ASD (Table 2). On the other hand, in the family-based study with the *HTR1B* gene, we observed undertransmission of the haplotype containing the alleles –161G/–261A ($P=0.003$) (Table 3). The power for this haplotype (GCAC) was calculated as 0.4986.

A significant positive interaction was detected between markers at *HTR1B* (–182 INS/*HTR1B*) and *HTR2C* (c.1078–31G>A) for the development of ASD in our set of patients ($P=0.005$). A total of 55 heterogeneity tests were performed. Applying this

Table 2 – Family-based analysis of haplotypes at HTR2C gene in ASD

Gene	Haplotypes [a]	Transmitted	Non transmitted	χ^2 (df)	*p*-value
HTR2C	1-G-G-G-A	29	29		
	3-G-G-G-A	122	113		
	4-G-G-G-A	15	12		
	1-G-A-C-G	8	17		
	Others [b]	36	39		
	Global test	210	210	4113 (4)	0.391

[a] Alleles are arranged following their genomic position [g.75GT (16_21); c.649+16895G>A; c.763+30G>A;c.986G>C, c.1078–31G>A].
[b] Haplotypes with frequencies less of 1% were grouped for the analysis.

Table 3 – Transmission disequilibrium test results (TRANSMIT analysis) for *HTR1B*

Haplotypes [a]	Observed	Expected	χ^2 (df)	*p*-value
G-D-T-C	0.8876	0.5126	0.719 (1)	0.396
G-C-A-C	1.392	5.7951	9.820 (1)	0.003
G-C-A-G	51.669	60.005	3.476 (1)	0.062
G-D-A-C	0.1603	0.67342	1.047 (1)	0.306
G-D-A-G	10.391	9.4158	0.259 (1)	0.611
G-C-T-G	146.46	139.28	1.356 (1)	0.244
G-C-T-C	2.1687	1.6689	0.863 (1)	0.353
T-C-A-G	140.38	141.44	0.029 (1)	0.865
T-D-A-C	1.0307	1.759	0.854 (1)	0.355
T-D-A-G	3.2424	2.1296	1.779 (1)	0.182
T-C-T-C	2.2383	1.8872	0.545 (1)	0.460
T-C-A-C	134.84	130.33	0.602 (1)	0.438
T-C-T-G	8.8364	8.3552	0.097 (1)	0.755
T-D-T-C	0.29899	0.75019	0.760 (1)	0.383
Global test			15.011 (7) [b]	**0.036**

[a] Alleles are arranged in the following order: –161T>G; –182 INS/DEL-181, –261 A>T; c.861G>C. The INDEL modification is coded 'C' for insertion and 'D' for deletion. Deletions were not found in homozygosis in our sample.
[b] Haplotypes with frequencies less of 1% were grouped for the analysis with TRANSMIT.

Table 4 – Genomic information and sequences of PCR primer pairs used for amplification of the polymorphisms selected for the study

Gene/ chromosomal location	Sequence variant	NCBI_ID	PCR product length (bp)	Annealing temperature (C°)	Forward primer	Reverse primer
HTR1B 6q13	–161T>G [a]	rs11568817				
	–182INS/ DEL-181 [a]	rs130057				
	–261A>T [a]	rs130058	394	57.0	GCAAGCTTTGGTCTCTACACCT	TTTGTCCCCAGTTGATAGTTCC
	c.861G>C	rs6296	387	57.0	GACCACATCCTCTACACGGTCT	GATGAAGAAGGGTAGCCAACAC CCGGATCTCCTGTGTATGT [b]
HTR2C	g.75GT (16_22) [c]	–		57.0	CTTGAAGGGAGTTTCAAAGC	CCGGTCTCTTAGTGCATCTG
Xq24	c.649 +16895G>A	–	313	52.0	AAGCAATGGGTTCTGAGATGTT	AGTTACTGAGCTGCCTGGATCT GGATGTTACTACTTATTATTTTAGT [b]
	c.763 +30G>A	rs2248440	446	50.8	GCTCTCTTTGCCATATTTTATC	TTGAATAGGAAACACCCATAAT CCAAGCAAAGTTATCTTTT [b]
	c.796G>C	rs6318	528	53.8	AAGCAGTTGTTTTGCATGAGC	CCTTCTGTCACACGATTTGCT GGCCTATTGGTTTGGCAAT [b]
	c.1078 –31G>A	rs5946005	513	52.2	TATATGTCACGCTGAAGGTATC	ACTAAATAAAAGAACCCGATCA GTCAGTACAATTTGGATGAC [b]

[a] Variants in the promoter region of the *HTR1B* gene were genotyped using the same primer pair.
[b] Single primers designed for genotyping using SNuPE method.
[c] Reference sequence for microsatellite g.75GT(16_22), in the promoter region of *HTR2C*, is U49648.

number to the usual 0.05 critical level of significance after a Bonferroni's correction, we obtain a corrected alpha level of about 0.001.

3. Discussion

Our main aims in this study were to explore the possible involvement of two serotonin receptor genes in the etiology of ASD in case-control and family-based studies. Based on the case-control and TDT results, we considered that markers at *HTR2C* are not relevant to the predisposition to ASD in our population. However, we observed a trend of undertransmission of *HTR1B* haplotypes including alleles –161G/–261A in our set of ASD patients.

It has been shown that the combination of the alleles –161G/–261A at *HTR1B* confers 2.3 fold higher levels of protein expression than the alleles –161G/–261T (Duan et al., 2003). Therefore, it is possible that lower expression levels of *HTR1B* receptor are associated with the development of ASD in a small proportion of Brazilian patients. Considering that the allelic frequencies of these SNPs at *HTR1B* significantly differ between individuals of European and African ancestry (data available under request), the negative result observed in our case-control study for SNPs at HTR1B and ASD can be due to stratification, as Brazilian population is ethnically admixed (Salzano and Bortolini, 2002).

The *HTR1B* alleles (–161G/–261A) identified as undertransmitted in ASD are different to those identified in the interaction between *HTR1B* (–182 INS) and *HTR2C* (c.1078–31G>A), therefore we did not consider this interaction as a relevant result. This finding can also reflect population stratification as the analysis to measure interaction does not take into account this possible confounding effect.

5-HT_{1B} can be transiently co-expressed with *SLC6A4* in the IV layer thalamocortical axons in rat neurodevelopment (Boylan et al., 2000) and can form heterodimers with 5-HT_{1D}, (Xie et al., 1999), which are genes also found to be associated with ASD (Coutinho et al., 2007; Conroy et al., 2004; Coutinho et al., 2004; McCauley et al., 2004; Guhathakurta et al., 2008). Therefore, our results taken together with the functional roles of *HTR1B* and that it is mapped in a candidate region for autism (6p13) (Philippe et al., 1999), has led us to consider *HTR1B* a predisposing locus for ASD. Although the data on *HTR2A* and ASD is controversial (Veenstra-VanderWeele et al., 2002; Cho et al., 2007), *HTR1B* would represent the fourth gene in the serotonergic pathway found to be associated to autism (Cho et al., 2007, Coutinho et al., 2007, Guhathakurta et al., 2008), further reinforcing the importance of this system to the etiology of autism.

4. Experimental procedures

4.1. *Subjects*

A total of 252 males (average age: 11.68 ± 6.13) diagnosed with ASD were recruited for this research. Most of them were ascertained at the Instituto de Psiquiatria (IPq) of the Hospital das Clínicas (HC), Universidade de São Paulo, São Paulo. All patients were diagnosed by experienced psychiatrists using DSM-IV and ICD-10. Children's behavior was characterized at age of four and score questions of ADI-R (Lord et al., 1994) were applied.

Patients who matched the cutoffs for diagnosis of autism according to the ADI-R were included in our sample with the narrow diagnosis of autism. Those matching the triad but without delayed speech were classified as Asperger Syndrome and patients who did not fill the ADI-R score criteria for one area of interest in three were considered as having Pervasive

Developmental Disorders Not Otherwise Specified (PDDNOS). Of the 252 patients, 194 were autism, 37 Asperger and 20 PDDNOS.

We excluded patients with known genetic conditions associated with ASD such as Angelman syndrome and tuberous sclerosis, metabolic disorders, chromosomal abnormalities, or who had a drug-exposure history, teratogenic medication or infectious diseases. All males were excluded for the Fragile X syndrome.

The control group used for case-control study was recruited at the Hospital das Clinicas and, like our subjects, consisted of Brazilians of European ancestry, mainly from Portugal, Spain, Italy and Germany.

This project was approved by the Ethics Committee of the Institutes where the study was conducted. Patients were included only after a signed written informed consent by the parents or a guardian.

4.2. *Genotyping*

Blood samples were collected from patients, their parents and siblings (whenever available) and DNA extraction was carried out by the salt method and AUTOPURE LS equipment (Gentra system).

We analyzed the following SNPs in the *HTR1B* and *HTR2C* genes: –161T>G (rs11568817:T>G), –182 INS/DEL-181 (rs130057), –261A>T (rs130058:A>T), c.861G>C (rs6296:G>C) at *HTR1B* and g.75GT(16_21)(U49648), c.649+16895G>A (identified by sequence analysis of the snoRNA *HBI-36* gene), c.763+30G>A (rs2248440), c.986G>C(rs6318) and c.1078–31G>A (rs5946005) at *HTR2C*. Polymorphism nomenclature, genomic characteristics and primers designed for analysis of the polymorphisms selected for this study are available in Table 4 and their location are illustrated in Figs. 1 and 2 (Supplementary material). Five SNPs (c.861G>C; c.649+16895G>A; c.763+30G>A; c.986G>C; c.1078–31G>A) were genotyped using SNuPE® (Single Nucleotide Primer Extension, GE Healthcare™). Two functional SNPs (–161T>G; –261A>T) and an INS/DEL modification –182 INS/DEL-181 in the promoter region of *HTR1B* were genotyped by sequencing. The microsatellite g.75GT(16_21) at the promoter region of *HTR2C* gene was genotyped using FAM labeled primers flanking the repeat (GenBank accession number U49648) and analyzed with MegaBACE Genetic Profiler Software 2.2. Except for –182 INS/DEL-181/*HTR1B* and c.763 +30G>A/*HTR2C*, all the others SNPs are in linkage disequilibrium (data available under request).

4.3. *Statistical analysis*

For case-control haplotype analysis of the *HTR2C* gene, which maps to the X chromosome, we applied the methodology developed by Dr. Paulo Otto, as detailed in the Supplementary material and methods (Appendix 1). SNPalyse v.6 and INSTAT were also used in case-control analysis of autosomal haplotypes and individual markers. TDT tests were performed using TRANSMIT v.2.5.4 (Clayton, 1999). To evaluate the power of the samples to detect undertransmission of haplotypes we used the program PS Power and Sample Size Calculation Version 2.1.30, 2003 (Dupont and Plummer, 1997). The Chi-square test (χ^2) was used for Hardy–Weinberg Equilibrium analysis.

In order to investigate the possibility of interaction effects of individual markers for the etiology of ASD, we also analyzed the existence of association between all possible pairs of markers from 8 loci studied through chi-squared heterogeneity tests performed on contingency tables. For each pair of loci, linked or unlinked, contingency tables were constructed for the corresponding haplotypes (in the case of linked loci) or gene combinations (in the case of unlinked loci) among controls (group 0) and patients (group 1). A third contingency table was constructed for housing data pooled from both groups (group 2=0+1). A heterogeneity chi-squared (HCS) value was obtained from HCS=CS0+CS(1)–CS(2), with a number of degrees of freedom calculated after $df(H)=df(0)+df(1)-df(0+1)$. A significant chi-squared heterogeneity test value indicates that the association level between markers is different among controls and patients. The microsatellite was excluded from this analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2008.11.007.

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