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ORIGINAL INVESTIGATION

Evidence for epistasis between *SLC6A4* and *ITGB3* in autism etiology and in the determination of platelet serotonin levels

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Abstract Autism is a neurodevelopmental disorder of unclear etiology. The consistent finding of platelet hyperserotonemia in a proportion of patients and its heritability within affected families suggest that genes involved in the serotonin system play a role in this disorder. The role in autism etiology of seven candidate genes in the serotonin metabolic and neurotransmission pathways and mapping to autism linkage regions (*SLC6A4*, *HTR1A*, *HTR1D*, *HTR2A*, *HTR5A*, *TPH1* and *ITGB3*) was analyzed in a sample of 186 nuclear families. The impact of interactions among these genes in autism was assessed using the multifactor-dimensionality reduction (MDR) method in 186 patients and 181 controls. We further evaluated whether the effect of specific gene variants or gene interactions associated with autism etiology might be mediated by their influence on serotonin levels, using the quantitative transmission disequilibrium test (QTDT) and the restricted partition method (RPM), in a sample of 109 autistic children. We report a significant main effect of the *HTR5A* gene in autism ($P = 0.0088$), and a significant three-locus model comprising a synergistic interaction between the *ITGB3* and *SLC6A4* genes with an additive effect of *HTR5A* ($P < 0.0010$). In addition to the previously reported contribution of *SLC6A4*, we found significant associations of *ITGB3* haplotypes with serotonin level distribution ($P = 0.0163$). The most significant models contributing to serotonin distribution were found for interactions between *TPH1* rs4537731 and *SLC6A4* haplotypes ($P = 0.002$) and between *HTR1D* rs6300 and *SLC6A4* haplotypes ($P = 0.013$). In addition to the significant independent effects, evidence for interaction between *SLC6A4* and *ITGB3* markers was also found. The overall results implicate *SLC6A4* and *ITGB3* gene interactions in autism etiology and in serotonin level determination, providing evidence for a common underlying genetic mechanism and a molecular explanation for the association of platelet hyperserotonemia with autism.

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Introduction

Autism (AD; OMIM 209850) is a heritable complex neurodevelopmental disorder characterized by impairments in social interaction and communication, and restricted and stereotyped patterns of interests and behaviors (Lord et al. 2000). Although the etiology of autism is not understood, several lines of evidence indicate that the serotonin (5-HT) system may play an important role. Serotonin is a monoamine neurotransmitter involved in the regulation of biological functions such as emotional behavior, sleep, pain sensitivity and hormone release, and plays a crucial role in synaptogenesis and brain development. One of the few consistent findings in autism is platelet hyperserotonemia in a proportion of patients. Platelet serotonin levels have been shown to be highly heritable, both in healthy and in autistic individuals (Abney et al. 2001; Ober et al. 2001; Coutinho et al. 2004). It is therefore plausible that the increased serotonin levels found in autism are genetically determined, and thus the quantification in platelets reflects the levels both in the mature central nervous system (CNS) and during development, at times when the brain may be particularly sensitive to abnormal neurotransmitter function. Increased levels of 5-HT and/or anomalous transmission during the prenatal and early postnatal period are hypothesized to result in abnormal synaptogenesis and brain development, with consequent behavioral manifestations that could be variable according to the brain area affected and to the timing of the insult, and which would persist throughout life in autistic individuals (Whitaker-Azmitia 2005). The genetic factors determining serotonin levels have not been fully established, but are likely candidate genes for autism etiology that can be identified as quantitative trait loci (QTLs) for serotonin levels and confirmed as autism susceptibility factors.

Multiple molecules involved in serotonin metabolism and neurotransmission are known to or have the capacity to regulate serotonin levels in the brain, and are often targets for genetic and pharmacological studies in autism. Some of these molecules are encoded by genes mapping to autism linkage regions. For instance, the serotonin transporter gene *SLC6A4* and the gene encoding the integrin $\beta 3$ subunit *ITGB3*, which were both identified as QTLs for serotonin levels (Weiss et al. 2004, 2005a), are located on 17q11.1-q12 and 17q21.32, respectively, under two linkage peaks for autism (Stone et al. 2004; Cantor et al. 2005). The serotonin receptor genes *HTR2A* on 13q14-q21 and *HTR5A* on 7q36.1, also map to regions identified by linkage screens for autism (Barrett et al. 1999; Bradford et al. 2001; Alarcón et al. 2002; Auranen et al. 2002). The *SLC6A4* gene has received particular attention, chiefly because the serotonin transporter is a target for selective serotonin reuptake inhibitor (SSRI) drugs that are effective in neuropsychiatric disorders. Various groups have reported the association of polymorphic markers within this gene with autism, including an insertion/deletion polymorphism in the promoter that regulates the serotonin reuptake rate (Lesch et al. 1996); however, the associated alleles were often not consistent among studies, and the results were not replicated in several other population samples (Devlin et al. 2005). In a previous study, we have shown that abnormally high platelet 5-HT levels in autistic children are partly explained by genetic variants in *SLC6A4*, although an association of this gene with autism could not be established in our population sample (Coutinho et al. 2004). This suggests that while *SLC6A4* could act as a major gene, other genes are very likely interacting with it in the determination of hyperserotonemia associated with autism. The observations were consistent with reported differences in narrow and broad heritability of 5-HT level estimates which suggest that more than one gene is influencing this trait in a nonadditive manner (Abney et al. 2001; Ober et al. 2001), and with the recent genome-wide scan results for whole blood 5-HT, which identified two QTLs on 17q, *SLC6A4* and *ITGB3*. Several other genes encoding molecules involved in the 5-HT system are plausible candidates for a role in the determination of serotonin levels. The 5-HT autoreceptors 5-HT1A and 5-HT1D play an inhibitory role in 5-HT neurotransmission, while a reduced number of platelet 5-HT2A binding sites is present in autistic children with high 5-HT levels and their parents (McBride et al. 1989; Cook et al. 1993), suggesting the involvement of specific variants of the genes encoding these receptors in hyperserotonemia in autistic patients. The 5-HT5A receptor gene *HTR5A* is mainly expressed in the CNS, also suggesting an important role in mediating the central effects of 5-HT. Tryptophan hydroxylase 1 (TPH1), which catalyzes the rate-limiting step in the synthesis of 5-HT, is located both in the periphery and in the CNS (Zill et al. 2005), and therefore alterations in the *TPH1* gene may be involved in any phenotype resulting from a dysfunction of the 5-HT system.

The multiplicity of possible players in the regulation of serotonin levels indicates that interactions between genes, or epistasis, are important in the definition of this phenotype. Epistasis is thought to occur to allow genetic buffering, i.e., to stabilize a phenotype through the requirement that several genes in a gene network

are mutated before a substantial effect on the phenotype takes place. It is therefore likely that epistasis is a ubiquitous component of the genetic architecture of complex traits, in which the correlations between genotype and phenotype are usually nonlinear not only because of genetic heterogeneity but also due to gene–environment and gene–gene interactions (Ritchie et al. 2001; Moore 2003b, 2005; Thornton-Wells et al. 2004). It is conceivable if not likely that epistasis in the absence of detectable independent effects of any gene may impact on a phenotype, thus significantly hampering the power of current statistical genetics methods to uncover the genetic basis of complex traits. A research approach that takes into account gene interactions may therefore be a more useful strategy for this purpose.

In the present study, we analyzed the role in autism etiology of seven candidate genes in the serotonin metabolic and neurotransmission pathways and searched for the occurrence of epistatic effects among them. For this purpose, we focused on genes with an established biological role in the serotonin pathway that were located near linkage peaks for autism or had been reported to influence serotonin level distribution, and selected mainly functional polymorphisms that might regulate serotonin levels. We further evaluated whether the impact of specific gene variants and gene interactions in autism etiology might be mediated by their influence on serotonin levels, thus seeking a better understanding of the importance of serotonin levels for the pathophysiology of this disorder and eventually for autism treatment.

Materials and methods

Subjects and clinical assessments

One hundred and eighty six autistic patients and their parents were recruited at the Hospital Pediátrico de Coimbra (HP), originating from mainland Portugal ($N = 171$) and the Azorean islands ($N = 15$). The male-to-female ratio was of 4.8:1, and the age ranged from 2 to 18 years (mean 6.8 years). Patients were diagnosed using DSM-IV criteria (American Psychiatric Association 1994), the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al. 1994) and the Childhood Autism Rating Scale (CARS) (Schopler et al. 1988). Idiopathic subjects were included after clinical assessment and screening for known medical and genetic associated conditions. Neuropsychological evaluation was performed using the Ruth Griffiths Mental Developmental Scale II (Griffiths 1984) or the Wechsler Intelligence Scale for Children (WISC 1974). Control adults consisted of 181 unrelated healthy blood donors (male:female ratio of 3.9:1) with no family history of neuropsychiatric diseases, recruited at the Centro Regional de Sangue de Lisboa and at the Hospital Egas Moniz. Control children consisted in 38 unrelated healthy individuals (male:female ratio of 1.3:1), recruited at the HP. The study was approved by the HP ethical committee, and all participants or legal representatives signed an informed consent.

Measurement of platelet serotonin levels

Platelet serotonin content was measured by high performance liquid chromatography (HPLC) in 109 autistic patients and their parents, as previously described (Coutinho et al. 2004). Serotonin levels were analyzed in subjects free of medication. In a previous study (Coutinho et al. 2004), we found no correlation of platelet serotonin levels with sex or age, and therefore these parameters were not used as covariates in the present analysis.

Genotyping

A blood sample from the patients, their parents, and control individuals was used for DNA extraction by salting out (Lahiri and Nurnberger 1991). Genotyping of polymorphisms in *SLC6A4*, *HTR2A*, *HTR1D*, *HTR2A*, *HTR5A*, and *TPH1* was based on previously described methods (see Supplementary Table S1). Two polymorphisms in the *HTR1A* gene (see Supplementary Table S1), were genotyped through the Amplifluor System™ (KBiosciences, UK) or by PCR-RFLP through insertion of a restriction site, as follows. The PCR (30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s) was performed with 25 ng of genomic DNA, 200 μM of each dNTP (Promega), 2 mM of $MgCl_2$ (Promega), 1 × PCR buffer (Promega), 1.5 U of Taq polymerase, and 0.4 μM of each primer (final volume of 12.5 μl). For rs6295 and rs878567, the polymorphisms were detected by restriction with *Taq*I (Roche) and *Ape*KI (New England Biolabs), respectively, according to the manufacturer's instructions, and resolved by electrophoresis in a 2.5% conventional agarose gel. Two polymorphisms in *ITGB3* (see Supplementary Table S1) were genotyped as follows. For rs5918, the PCR (30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) was performed with 37.5 ng of genomic DNA, 200 μM of each dNTP (Promega), 2 mM of $MgCl_2$ (Promega), 1 × PCR buffer (Promega),

1.5 U of Taq polymerase, and 0.36 μM of each primer (final volume of 12.5 μl). For rs15908, the PCR (30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s) was performed with 37.5 ng of genomic DNA, 200 μM of each dNTP (Promega), 2 mM of $MgCl_2$ (Promega), 1 × PCR buffer (Promega), 1.5 U of Taq polymerase, and 1.2 μM of each primer (final volume of 12.5 μl). For rs5918 and rs15908, the polymorphisms were detected by restriction with *Hpa*II (Fermentas) and *Taq*I (Roche) respectively, according to the manufacturer's instructions, and resolved by electrophoresis in a 2% conventional agarose gel.

Statistical analysis

The effects of each marker in association with autism etiology were assessed using the Extended Transmission Disequilibrium Test (ETDT, version 2.4) for individual markers (Sham and Curtis 1995) and TRANSMIT (Clayton 1999), version 2.5.2, for haplotypes. Testing for genetic interactions in association with autism was performed using the multifactor-dimensionality reduction (MDR) method (Ritchie et al. 2001; Moore 2004), version beta 1.0.0 RC1, available from http://www.epistasis.org/mdr.html. The details of the MDR method have been presented elsewhere (Ritchie et al. 2001; Moore 2004; Moore et al. 2006). Briefly, a set of n polymorphisms are selected. The n polymorphisms and their possible multilocus classes are represented in n-dimensional space; for example, for two loci with three genotypes each, there are nine possible two-locus genotype combinations. Then, the ratio for the number of cases to the number of controls is calculated within each multilocus class. Each multilocus class in n-dimensional space is then labeled as ''high-risk'' if the cases to controls ratio meets or exceeds some threshold (e.g., ≥1), or as ''low-risk'' if that threshold is not exceeded; the method thus reduces the n-dimensional space to one dimension with two levels (''low-risk'' and ''high-risk''). The process of creating a new variable as a function of two or more variables is called constructive induction (Moore et al. 2006). This new single MDR variable is assessed for its ability to classify subjects as sick or healthy using a naïve Bayes classifier. All possible combinations of polymorphisms are evaluated and the best selected as the model that is most likely to generalize to independent datasets as assessed by estimating a testing balanced accuracy (TBA) with ten-fold cross-validation. Balanced accuracy is defined as the arithmetic mean of sensitivity and specificity:

$$1/2\,(\mathrm{TP}/\mathrm{TP}+\mathrm{FN}+\mathrm{TN}/\mathrm{TN}+\mathrm{TP}) = (\text{sensitivity}+\text{specificity})/2$$

where TP are true positives, TN are true negatives, and FN are false negatives (Velez et al. 2006). Cross validation divides the data into a training set and a testing set in order to assess generalizability (Coffey et al. 2004). With ten-fold cross validation, the data are divided into ten equal parts, and the model is developed on 9/10 of the data (training set) and then tested on 1/10 of the remaining data (testing set). This is repeated for each possible 9/10 and 1/10 of the data, and the resulting ten testing accuracies averaged (Hastie et al. 2001). In addition to the testing accuracy, we also report the cross validation consistency (CVC) that is a measure of how many times out of ten divisions of the data that MDR found the same best model (Moore 2003a). Models that are true positives are likely to generalize to independent datasets and will have estimated testing accuracies of greater than 0.5. Permutation testing was performed to assess the probability of obtaining a testing accuracy as large or larger than observed in the original data given the null hypothesis of no association is true. This is carried out by randomizing the case-control labels 1,000 times and repeating the MDR analysis on each randomized dataset. This process yields an empirical distribution of testing balanced accuracies under the null hypothesis that is in turn used to calculate a P value. Permutation testing is important to correct for multiple testing (Good 2000). MDR was run for models with two and three marker combinations between the individual markers or haplotypes. Missing genotypes were imputed with MDR data tool software (beta version 0.4.3), which allows the global replacement of unknown values and the imputation of the data from a model constructed by the software from the existing dataset. Unknown haplotypes were estimated with the program PHASE (Stephens et al. 2001), version 2.1.1. The population sample used for the MDR test consisted of 186 autistic children and 181 healthy adult controls. Using approximately the same number of cases and controls allows for an increase in power; for this analysis, the ratio cases/controls was 1.03. Genotypic combinations for which the ratio cases/controls was equal to 1.03 were set to be classified as unknown risk. The models presenting the maximum TBA were selected as the best MDR models.

To confirm, visualize, and interpret the genetic interactions identified, MDR allows the construction of interaction dendograms (Moore et al. 2006). This graph plots the interactions between the variables

tested (determined by interaction entropy analysis) in hierarchical clustering, placing the most strongly interacting variables close together at the leaves of the tree. Interaction entropy is a measure of information gain (i.e., percentage of entropy removed) on case-control status from merging two variables together over that provided by the two variables independently. Additive and nonadditive interactions, as well as redundancy, can thus be easily visualized and used to interpret the MDR models.

Statistical analysis of platelet 5-HT levels was performed following logarithmic (ln) transformation of the quantitative variable, in order to approach a normal distribution. Analysis with haplotypes was performed only when it was possible to establish phase. To test the main effect of each marker on the 5-HT distribution, quantitative transmission disequilibrium tests (QTDT) were performed, implemented in the QTDT software, version 2.4.6 (Abecasis et al. 2000). The orthogonal model of association was considered, including variance components (individual and nuclear family environment and a polygenic component). This test, based on maximum-likelihood estimates, allows variance components testing of family-based samples for association and transmission disequilibrium, estimating the relative weight of each contributor to the phenotype. Association is partitioned into between- and within-family components, the test for the latter being free of confounding population substructure effects, regardless of the nuclear family composition. The broad heritability of platelet serotonin levels was determined using QTDT, by testing the significance of the polygenic variance component of the trait distribution; a null model including solely an environmental component of variance was compared to a full model including this and a polygenic variance component. Genetic interactions in the determination of platelet 5-HT levels were tested using the restricted partition method (RPM) (Culverhouse et al. 2004), in a sample of 109 autistic children and 38 age-matched controls. RPM is a model-free method for detecting and characterizing nonadditive interactions among discrete genetic and environmental factors that contribute to quantitative trait variation. The goal of RPM is to find partitions of multilocus genotypes that explain a significant proportion of the observed trait variation. For each model, a coefficient of determination (R^2) is estimated for the quantitative trait value regressed on the final genotype groups, and is a measure of the proportion of the total variance explained by the groups of the respective model. We ran RPM for two-way interactions between the 11 individual markers and haplotypes, in a total of 97 tests performed. The significance of each model was validated with 9,700 permutations, according to the number of tests (number of permutations = [1/(0.05/number of tests)] × 5), in order to generate a large enough empirical null distribution to yield a P value with the desired precision ($\alpha = 0.05$). In the permutation test, the data trait values are randomized and, for each permutated data, RPM is performed and the R^2 is estimated. The significance of each model is assessed by the frequency with which the R^2 from the original data exceeds the permutated R^2 values. Only groups with more than one individual were considered in the analysis. Due to the small sample size, no more than two-way interactions were tested.

Results

To assess individual contributions of each marker in autism etiology, the TDT was carried out for 11 individual markers in the seven candidate genes and their respective haplotypes (Tables 1, 2). A significant main effect in autism was found for *HTR5A* rs1800883 marker ($P = 0.0088$), with allele –19G significantly overtransmitted to the autistic probands (Table 1). A specific haplotype of *HTR5A* markers containing the undertransmitted –19C allele was significantly less transmitted than expected ($P = 0.0333$) (Table 2). No significant associations were found with other individual markers or haplotypes.

The MDR method was used to assess the impact of two- and three-way combinations between the 11 individual markers in autism etiology (Table 3). The best two-marker model selected by MDR was an interaction between *SLC6A4* intron 2 VNTR and *ITGB3* rs5918, with a maximum testing balanced accuracy (TBA) of 0.6357, thus being able to classify correctly 64% of the individuals used in the analysis ($P < 0.001$), and with a cross-validation consistency (CVC) of 6/10. The best three-marker model identified included these two markers and added *HTR5A* rs6320, increasing the TBA to 67% ($P < 0.001$), and with a CVC of 4/10. A two-way model between *SLC6A4* and *ITGB3* marker haplotypes was also significant, with a TBA of 0.5853 ($P = 0.020$), but with a CVC of 9/10. The best three-way marker haplotype model again included markers in *SLC6A4*, *ITGB3*, and *HTR5A* (TBA = 0.6066, $P = 0.009$ and CVC of 10/10). This indicates that interaction between genetic variants at *SLC6A4* and *ITGB3* genes is associated with increased risk for autism, a finding compatible with the linkage peaks for autism on chromosome 17q, in the regions where these genes map (Stone et al. 2004; Cantor et al.

Table 1 Transmission *disequilibrium* test results (ETDT analysis), to assess the main effects of each marker in association with autism etiology

Marker	Allele	T	NT	$\chi 2$ (*df*)	*P* value
TPH1 rs4537731	–6526G	85	82	0.054 (1)	0.8164
	–6526A	82	85	0.054 (1)	0.8164
	Global test			0.054 (1)	0.8164
HTR2A rs6311/rs6313	–1438A/102T	72	71	0.007 (1)	0.9334
	–1438G/102C	71	72	0.007 (1)	0.9334
	Global test			0.007 (1)	0.9333
HTR1D rs6300	1080T	33	26	0.831 (1)	0.3622
	1080C	26	33	0.831 (1)	0.3622
	Global test			0.832 (1)	0.3616
SLC6A4 5-HTTLPR	L	71	71	0.000 (1)	1.0000
	S	71	71	0.000 (1)	1.0000
	Global test			0.000 (1)	1.0000
SLC6A4 intron 2 VNTR	Stin2.9	2	1	not tested	
	Stin2.10	70	52	2.656 (1)	0.1032
	Stin2.12	53	72	2.888 (1)	0.0893
	Global test			3.005 (2)	0.2226
ITGB3 rs5918	Leu33	43	35	0.821 (1)	0.3651
	Pro33	35	43	0.821 (1)	0.3651
	Global test			0.822 (1)	0.3647
ITGB3 rs15908	1143A	67	76	0.566 (1)	0.4517
	1143C	76	67	0.566 (1)	0.4517
	Global test			0.567 (1)	0.4516
HTR1A rs6295	–1019C	88	82	0.212 (1)	0.6454
	–1019G	82	88	0.212 (1)	0.6454
	Global test			0.212 (1)	0.6454
HTR1A rs878567	1556T	81	90	0.474 (1)	0.4913
	1556C	90	81	0.474 (1)	0.4913
	Global test			0.474 (1)	0.4912
HTR5A rs1800883	–19G	81	51	6.818 (1)	0.0091
	–19C	51	81	6.818 (1)	0.0091
	Global test			6.878 (1)	0.0088
HTR5A rs6320	12A	74	69	0.175 (1)	0.6759
	12T	69	74	0.175 (1)	0.6759
	Global test			0.175 (1)	0.6758

T transmitted, *NT* not transmitted

2005). Three-way genetic combinations between these two genes and *HTR5A* are also associated with increased risk for autism, in agreement with the main effect in autism found for *HTR5A* in this sample (see Supplementary Table S2 for a detailed description of the genotypic combinations associated with high- and low-risk for autism for the MDR best models).

The interaction dendograms shown in Fig. 1a, b allow a better interpretation of the genetic effects identified by MDR. As expected, the strongest synergy among individual markers (Fig. 1a, line 1) was found between *SLC6A4* intron 2 VNTR and *ITGB3* rs5918, indicative of a nonadditive effect. Synergy was moderate for markers *HTR1A* rs6295 and *HTR5A* rs6320 (Fig. 1a, line 2); however, this model was not good in predicting disease-risk status for autism (CVC of 0/10), and was not selected by the MDR analysis. Importantly, the Fig. 1a interaction dendogram shows that for the best three-marker model associated with autism, *SLC6A4* intron 2 VNTR and *ITGB3* rs5918 act synergistically but *HTR5A* rs6320 contributes with an additive effect to the model (Fig. 1a, line 3). This interpretation is consistent with the individual association of *HTR5A* with autism. Figure 1b corroborates these results, displaying a strong synergy between *SLC6A4* and *ITGB3* marker haplotypes (line 1) but an additive effect of *HTR5A* haplotypes (line 3).

Our sample provides a very significant evidence of a polygenic component ($P = 5 \times 10^{-7}$) for platelet serotonin levels, for which the broad heritability was estimated at 64%. The individual contributions of candidate gene marker alleles and haplotypes to platelet serotonin levels in autistic children were assessed using QTDT. In addition to the previously re-

Table 2 Transmission *disequilibrium* test results (TRANSMIT analysis), to assess the main effects of each marker haplotype in association with autism etiology

Marker	Haplotype	Observed	Expected	$\chi 2$ (*df*)	*P* value
SLC6A4 5-HTTLPR/intron 2 VNTR	L/Stin2.9	3.144	1.654	2.961 (1)	0.0853
	S/Stin2.9	0.045	0.563	1.074 (1)	0.3000
	L/Stin2.10	98.052	88.320	3.304 (1)	0.0691
	S/Stin2.10	35.701	34.560	0.107 (1)	0.7440
	L/Stin2.12	82.287	90.952	2.568 (1)	0.1090
	S/Stin2.12	120.770	123.950	0.295 (1)	0.5868
	Global test			8.502 (5)	0.1306
ITGB3 rs5918/rs15908	Leu33/1143A	169.860	171.110	0.045 (1)	0.8325
	Pro33/1143A	5.142	5.945	0.234 (1)	0.6285
	Leu33/1143C	87.142	80.438	1.546 (1)	0.2137
	Pro33/1143C	35.858	40.510	1.259 (1)	0.2618
	Global test			2.332 (3)	0.5065
HTR1A rs6295/rs878567	–1019C/1556T	3.508	6.119	2.478 (1)	0.1155
	–1019G/1556T	160.760	162.570	0.075 (1)	0.7842
	–1019C/1556C	185.160	180.540	0.494 (1)	0.4821
	–1019G/1556C	10.570	10.767	0.011 (1)	0.9159
	Global test			3.751 (3)	0.2897
HTR5A rs1800883/rs6320	–19G/12A	104.150	102.110	0.120 (1)	0.7286
	–19C/12A	4.501	4.186	0.056 (1)	0.8127
	–19G/12T	142.900	132.490	2.551 (1)	0.1102
	–19C/12T	94.446	107.210	4.532 (1)	0.0333
	Global test			4.825 (3)	0.1851

Table 3 Best models assessed by the MDR method for two-way and three-way combinations, to test for epistasis in association with autism etiology

Nb. markers	Best models			Cross-validation consistency	Testing balanced accuracy (Empirical *P* value)	Odds-ratio (95% Confidence Interval)
	Marker 1	Marker 2	Marker 3			
2	*SLC6A4* intron 2 VNTR	*ITGB3* rs5918		6/10	0.6357 (<0.001)	3.24 (2.12–4.97)
2	*TPH1* rs4537731	*HTR5A* rs6320		4/10	0.6348 (<0.001)	3.36 (2.18–5.17)
3	*SLC6A4* intron 2 VNTR	*ITGB3* rs5918	*HTR5A* rs6320	4/10	0.6676 (<0.001)	4.84 (3.11–7.54)
3	*HTR2A* rs6311/rs6313	*SLC6A4* intron 2 VNTR	*HTR5A* rs6320	4/10	0.6462 (<0.001)	4.68 (3.01–7.27)

ported contribution of *SLC6A4* haplotypes (Coutinho et al. 2004), we found significant associations of *ITGB3* rs5918 marker and rs5918/rs15908 haplotypes with serotonin level distribution (Table 4). The rs5918 marker allele Pro33 was positively associated with 5-HT levels in autistic children ($P = 0.0055$), accounting for 16.9% of the phenotypic variance, replicating previous findings of an association of this allele with 5-HT levels towards the high end of the distribution in a healthy population (Weiss et al. 2004, 2005a, 2005b). A significant global association was also observed for *ITGB3* rs5918/rs15908 haplotypes ($P = 0.0163$), with haplotype Pro33/1143C showing a significant positive association with the trait ($P = 0.0019$). The proportion of variance accounted for by this haplotype, estimated at 24.5%, was increased in relation to the independently associated rs5918 marker. Altogether, these results demonstrate significant main individual effects of *SLC6A4* and *ITGB3* in the determination of platelet 5-HT levels, which account for a significant proportion of the genetic trait variation in an autistic population. No associations were found with the remaining markers tested.

Interaction effects among the candidate gene markers contributing to the determination of 5-HT levels were modeled using RPM. Interactions were tested for all possible two-way marker and haplotype combinations, and are shown in Table 5 (see Supplementary Table S3 for detailed information about the genotypic combinations of the significant RPM

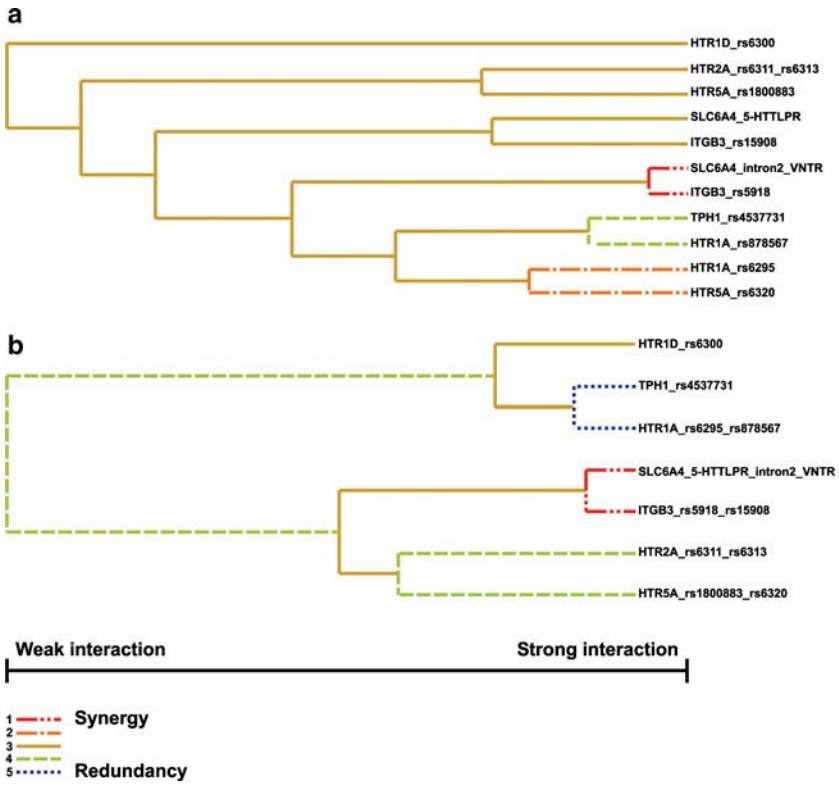

Fig. 1 Interaction dendograms for the markers tested in the MDR analysis for association with autism. **a** Interactions between the 11 individual markers, clearly showing a strong synergistic effect (epistasis) between *SLC6A4* intron 2 VNTR and *ITGB3* rs5918 (*line 1*); weaker synergy is found between *HTR1A* rs6295 and *HTR5A* rs6320 (*line 2*); redundancy is found for *TPH1* rs4537731 and *HTR1A* rs878567 markers (*line 4*); additivity lines (*line 3*) confirm the independent effect of the *HTR5A* rs6320 marker over *SLC6A4* intron 2 VNTR and *ITGB3* rs5918. **b** Interactions between haplotypes, where the strong synergy between *SLC6A4* and *ITGB3* and the additive effect of *HTR5A* with these two genes are confirmed; redundancy between markers is shown by *lines 4* and *5*

models). The analysis was restricted to two-way combinations because, given the present sample size, when testing higher-order interactions we would expect many two-locus genotypes to be void or have very few observations. The most significant models contributing to 5-HT distribution were found for interactions between *TPH1* rs4537731 and *SLC6A4* haplotypes ($P = 0.002$) and between *HTR1D* rs6300 and *SLC6A4* haplotypes ($P = 0.013$). Most interestingly, interactions between *SLC6A4* haplotypes and the *ITGB3* markers rs5918 ($P = 0.037$) and rs15908 ($P = 0.035$) also significantly contribute to trait distribution. Interaction between marker haplotypes in both genes was not significant, likely because the number of individuals used in the analysis of this particular model was lower ($N = 97$), with a consequent loss of statistical power. Other significant interactions were found in association with 5-HT levels, namely *ITGB3* rs5918 with *HTR5A* rs6320 ($P = 0.027$), and *ITGB3* haplotypes with *HTR1A* haplotypes ($P = 0.030$).

Because we initially hypothesized that a common set of genes or gene interactions might bear an impact in autism by contributing to the determination of 5-HT levels, we tested whether the interaction models identified as contributing the most to serotonin distribution using RPM might also be associated with autism. For this purpose, we analyzed the TBA and CVC of interaction models tested by MDR but not selected as best models. We found that two of the interaction models most significantly contributing to 5-HT distribution were also associated with autism, namely the interaction of *TPH1* rs4537731 with *SLC6A4* haplotypes (TBA = 0.5941, $P = 0.015$) and the interaction of *ITGB3* rs5918 with *HTR5A* rs6320 (TBA = 0.5923, $P = 0.025$); however for both these models the CVC was 0/10, and analysis of the corresponding interaction dendograms indicates that there is redundancy and additivity, respectively, between the marker variants. The other interaction models significantly contributing to 5-HT determination were not significantly associated with autism, except for those that included *SLC6A4* and *ITGB3* markers, as already referred.

The overall results therefore consistently implicate the *SLC6A4* and *ITGB3* genes in autism etiology and in serotonin level determination and show that interaction between the same genetic variants is associated with both traits, strongly suggesting a causal relationship.

Discussion

The main purpose of the present paper was to further our understanding of the role of serotonin pathway genes in autism. We report a significant main effect of the *HTR5A* gene in autism, with a significant associa-

Table 4 QTDT orthogonal tests of association with variance components, to assess the main effects of each marker and respective haplotypes in association with platelet serotonin levels

Marker	Allele	$\chi 2$ (*df*)	*P* value	Direction of association	Proportion of phenotypic variance accounted by each allele/haplotype (%)
ITGB3 rs5918	Leu33	7.71 (1)	0.0055	Negative	16.9
	Pro33	7.71 (1)	0.0055	Positive	16.9
	Global test	7.71 (1)	0.0055		
ITGB3 rs15908	1143A	1.57 (1)	>0.1		
	1143C	1.57 (1)	>0.1		
	Global test	1.57 (1)	0.2108		
ITGB3 rs5918/rs15908	Leu33/1143A	2.31 (1)	>0.1		
	Leu33/1143C	0.37 (1)	>0.1		
	Pro33/1143A	Not tested	Not tested		
	Pro33/1143C	9.68 (1)	0.0019	Positive	24.5
	Global test	10.29 (3)	0.0163		
SLC6A4 5-HTTLPR	L	5.72 (1)	0.0168	Positive	11.2
	S	5.72 (1)	0.0168	Negative	11.2
	Global test	5.72 (1)	0.0168		
SLC6A4 intron 2 VNTR	Stin2.10	3.92 (1)	0.0477	Positive	8.3
	Stin2.12	3.91 (1)	0.0480	Negative	8.5
	Global test	3.95 (1)	0.0468		
SLC6A4 5-HTTLPR/intron 2 VNTR	L/Stin2.10	10.34 (1)	0.0013	Positive	18.4
	L/Stin2.12	0.00 (1)	>0.1		
	S/Stin2.10	1.16 (1)	>0.1		
	S/Stin2.12	4.10 (1)	0.0429	Negative	9.1
	Global test	12.08 (3)	0.0168		
TPH1 rs4537731	–6526G	0.43 (1)	>0.1		
	–6526A	0.43 (1)	>0.1		
	Global test	0.43 (1)	0.5128		
HTR1A rs6295	–1019C	0.66 (1)	>0.1		
	–1019G	0.66 (1)	>0.1		
	Global test	0.66 (1)	0.4150		
HTR1A rs878567	1556T	0.09 (1)	>0.1		
	1556C	0.09 (1)	>0.1		
	Global test	0.09 (1)	0.7687		
HTR1A rs6295/rs878567	–1019C/1556T	Not tested	Not tested		
	–1019C/1556C	0.11 (1)	>0.1		
	–1019G/1556T	0.28 (1)	>0.1		
	–1019G/1556C	Not tested	Not tested		
	Global test	0.68 (3)	0.7135		
HTR1D rs6300	1080T	0.28 (1)	>0.1		
	1080C	0.28 (1)	>0.1		
	Global test	0.28 (1)	0.5941		
HTR2A rs6311/rs6313	–1438A/102T	1.31 (1)	>0.1		
	–1438G/102C	1.31 (1)	>0.1		
	Global test	1.31 (1)	0.2523		
HTR5A rs1800883	–19G	0.63 (1)	>0.1		
	–19C	0.63 (1)	>0.1		
	Global test	0.63 (1)	0.4279		
HTR5A rs6320	12A	0.47 (1)	>0.1		
	12T	0.47 (1)	>0.1		
	Global test	0.47 (1)	0.4926		
HTR5A rs1800883/rs6320	–19G/12A	0.09 (1)	>0.1		
	–19G/12T	0.01 (1)	>0.1		
	–19C/12A	Not tested	>0.1		
	–19C/12T	0.00 (1)	>0.1		
	Global test	1.20 (3)	0.7531		

Table 5 RPM results for two-way interactions, to test for epistasis in the determination of platelet serotonin levels

Two-way model		*N*	Nb. groups	R^2	Empirical *P* value
Marker 1	Marker 2				
TPH1 rs4537731	*SLC6A4* 5-HTTLPR/intron 2 VNTR	122	3	0.251	0.002
HTR1D rs6300	*SLC6A4* 5-HTTLPR/intron 2 VNTR	122	2	0.161	0.013
SLC6A4 5-HTTLPR	*SLC6A4* intron 2 VNTR	138	2	0.099	0.018
TPH1 rs4537731	*SLC6A4* 5-HTTLPR	140	2	0.097	0.019
ITGB3 rs5918	*HTR5A* rs6320	140	2	0.070	0.027
HTR1A rs6295/rs878567	*ITGB3* rs5918/rs15908	95	2	0.193	0.030
ITGB3 rs15908	*SLC6A4* 5-HTTLPR/intron 2 VNTR	115	2	0.209	0.035
HTR1D rs6300	*SLC6A4* 5-HTTLPR	139	2	0.065	0.036
ITGB3 rs5918	*SLC6A4* 5-HTTLPR/intron 2 VNTR	122	2	0.150	0.037
SLC6A4 5-HTTLPR	*HTR5A* rs1800883	142	2	0.076	0.042
ITGB3 rs15908	*HTR1A* rs6295/rs878567	106	2	0.130	0.042
HTR1A rs878567	*SLC6A4* 5-HTTLPR/intron 2 VNTR	121	2	0.191	0.054
SLC6A4 5-HTTLPR	*HTR5A* rs6320	141	2	0.069	0.057
HTR1A rs6295	*SLC6A4* 5-HTTLPR/intron 2 VNTR	122	2	0.197	0.064
HTR1A rs6295	*ITGB3* rs5918	138	2	0.026	0.070
HTR2A rs6311/rs6313	*SLC6A4* 5-HTTLPR	140	2	0.077	0.071
SLC6A4 5-HTTLPR	*ITGB3* rs5918	140	2	0.060	0.071
HTR2A rs6311/rs6313	*HTR5A* rs1800883/rs6320	120	2	0.128	0.077
HTR1A rs6295	*ITGB3* rs15908	135	2	0.072	0.078
HTR5A rs1800883	*SLC6A4* 5-HTTLPR/intron 2 VNTR	120	2	0.151	0.078
HTR1A rs6295	*ITGB3* rs5918/rs15908	127	2	0.114	0.085
SLC6A4 5-HTTLPR	*HTR5A* rs1800883/rs6320	123	2	0.124	0.104
SLC6A4 5-HTTLPR	*ITGB3* rs15908	135	2	0.066	0.110
SLC6A4 5-HTTLPR/intron 2 VNTR	*HTR1A* rs6295/rs878567	90	2	0.223	0.120
SLC6A4 5-HTTLPR/intron 2 VNTR	*ITGB3* rs5918/rs15908	97	2	0.216	0.125
SLC6A4 5-HTTLPR	*HTR1A* rs6295	139	2	0.060	0.130
HTR5A rs6320	*SLC6A4* 5-HTTLPR/intron 2 VNTR	123	2	0.127	0.130
SLC6A4 5-HTTLPR/intron 2 VNTR	*HTR5A* rs1800883/rs6320	86	2	0.180	0.130
SLC6A4 5-HTTLPR	*HTR1A* rs878567	139	2	0.060	0.150
HTR5A rs1800883	*ITGB3* rs5918/rs15908	129	2	0.078	0.150
ITGB3 rs15908	*HTR5A* rs1800883	139	2	0.054	0.160
HTR2A rs6311/rs6313	*SLC6A4* 5-HTTLPR/intron 2 VNTR	125	2	0.176	0.160
HTR1A rs878567	*ITGB3* rs15908	136	2	0.062	0.170
ITGB3 rs5918/rs15908	*HTR5A* rs1800883/rs6320	101	2	0.077	0.340

Only results with R^2 above 0 and *P* value lower than 1.0 are shown

tion of a specific marker and one significantly under-transmitted haplotype. This gene maps to a linkage region previously identified for autism, which has been suggested to contain a locus associated with restrictive–repetitive behaviors (Alarcón et al. 2002; Auranen et al. 2002). The 5-HT5A receptor is mainly expressed in the CNS (Rees et al. 1994) but not much is known regarding its specific function. *HTR5A* knockout mice show increased locomotor and exploratory behavior in novel environments but no change in anxiety-related behaviors (Grailhe et al. 1999). Activation of this receptor in phase-shift studies also suggests a role in circadian rhythm (Sprouse et al. 2004), which is impaired in autistic patients with sleep problems (Richdale and Prior 1995). While intriguing, this result should be interpreted with caution. We did not correct for multiple testing, as for small sample sizes common procedures such as the Bonferroni correction, while reducing the frequency of Type I errors, greatly increase the probability of Type II errors, considerably increasing the likelihood of missing out on genes of small effect (Feise 2002). We therefore favor replication in a larger independent dataset to properly assess the relevance of this finding.

We further identified a significant two-way interaction between markers in *SLC6A4* and *ITGB3*, indicating that epistasis between variants in these two genes is associated with increased risk to autism, even though the same markers do not show an individual association with the disease. This result may illustrate an extreme case that could, however, be common for complex traits, in which testing for interaction between genes uncovers a genetic liability dependent on gene interaction even when the genes involved show no independent main effect on the phenotype. The most significant model associated with autism was a three-

way combination between *SLC6A4*, *ITGB3*, and *HTR5A*, with an increased testing balanced accuracy and correctly classifying 67% of the individuals tested. Interpretation of this model using an interaction dendogram shows a strong synergistic effect between *SLC6A4* and *ITGB3* markers, but an additive effect of *HTR5A*, consistent with the independent main effect found for *HTR5A* in association with autism.

Significant independent effects in the regulation of 5-HT levels were observed for markers at *SLC6A4* and *ITGB3*, as expected given our previous observation of an association of *SLC6A4* variants with serotonin distribution in this sample, and reports of these two genes as QTLs on chromosome 17q for whole blood serotonin (Weiss et al. 2004, 2005a, 2005b). Importantly, an overlapping linkage peak on 17q has been reported for autism (Stone et al. 2004; Cantor et al. 2005; Sutcliffe et al. 2005), pointing to the *SLC6A4* and *ITGB3* genes as prime candidates for autism etiology. Previous studies have reported the association of various polymorphisms in *SLC6A4* with autism, which we do not replicate in our population. A possible explanation is that our sample size is inadequate for detection of this effect, and/or that the tested loci are not causative but in somewhat weak linkage disequilibrium (LD) with the true causative mutation. However, as discussed in Coutinho et al. (2004), we favor an explanation for this discrepancy residing on the frequency of hyperserotonemia in different samples. Only 11.4% of our patients have hyperserotonemia. We therefore propose that while genetic variants associated with platelet hyperserotonemia are also contributing to autism susceptibility, these are not as frequent in our population. In this context, it would be interesting to find out whether hyperserotonemia is more frequent in samples in which the *SLC6A4* gene has been found associated with autism. The present study provides further evidence for a common genetic architecture underlying autism and regulation of serotonin levels by showing that the epistatic interaction of genetic variants of these two genes contributes to the determination of 5-HT levels and is associated with autism. Two very recent studies, in which association of *ITGB3* with autism and whole blood serotonin levels was reported (Weiss et al. 2006a) and it was suggested that *ITGB3* may interact with *SLC6A4* in mediating autism susceptibility (Weiss et al. 2006b) lend further support to our hypothesis. Notably, these were the single nonadditive gene interactions consistent for autism and serotonin distribution. Interpretation of the joint results for the autism and 5-HT phenotypes indicates that the *HTR5A* gene, independently or in combination with *SLC6A4* and *ITGB3*, is not a major factor in the regulation of serotonin levels and thus likely does not contribute to autism etiology through the same mechanism at the biological level. Other gene–gene interactions were significant for the determination of platelet serotonin distribution in our sample. For instance, the results suggest that the increased 5-HT reuptake associated with specific functional variants of the *SLC6A4* gene (Meltzer and Arora 1988; Lesch et al. 1996; Greenberg et al. 1999) could be reinforced by increases in the synthesis of 5-HT mediated by sequence alterations in the *TPH1* promoter (Sun et al. 2005). Evidence of a contribution to 5-HT level distribution was found for interactions involving *SLC6A4* and *HTR1D* or *HTR5A*, and also *ITGB3* and *HTR1A* or *HTR5A*. An involvement of these molecules was expected, given the roles of the encoded molecules in serotonin transmission or metabolism, but we found no evidence for a meaningful impact on the autistic phenotype.

The significant main effect and significant genetic interactions between candidate genes here reported cannot explain the entirety of trait variation. This is not surprising, as the serotonin system pathway includes a large number of components, and genetic interactions not detected in our sample, as well as gene–environment effects that were not accounted in the analysis, are likely contributors to these complex phenotypes. The power of our analysis to detect genetic effects with a minor contribution, however, was limited by the small sample size and thus replication in a larger independent sample is important.

By showing a common genetic architecture underlying the two traits, the results strongly suggest that, at least for a proportion of autistic subjects, a genetically determined increase in serotonin levels, with likely important consequences for brain development and function, contributes to autism etiology. We find independent and interaction effects of the *SLC6A4* and *ITGB3* genes to contribute to the full range of serotonin concentrations. Since only about 11.4% of our patients have hyperserotonemia, the majority of individuals tested fall in the normal portion of the 5-HT distribution, indicating that they have genotypes at either gene and/or gene combinations that will determine normal platelet serotonin levels. Only the minority of hyperserotonemic patients are likely to have the fatal combination of variants in the *SLC6A4* and *ITGB3* genes that will significantly contribute to a dysfunction in serotonin regulation, which is reflected in the increased platelet serotonin levels and may lead to autism. We suggest that while each gene independently strongly contributes to the 5-HT trait variation in the normal range, the interaction of specific delete-

rious variants of both genes will potentiate serotonin dysregulation, reflected in increased serotonin levels in the periphery and leading to brain dysfunction/disease. We cannot rule out that each gene does not independently contribute to autism in individuals with hyperserotonemia, but do find that the interaction has a stronger effect on the phenotype that can be detected even in a small sample of individuals in which hyperserotonemia is observed.

The biological mechanism, however, has yet to be determined. A role for the serotonin transporter is biologically sound, as this molecule is chiefly responsible for the removal of serotonin from the synapses. However, the association of specific transporter gene variants with autism has not been consistent among studies, even though it is clearly associated with serotonin level variation. Janusonis (2005) has established a theoretical model for serotonin release from gut enterochromaffin (EC) cells to the blood stream proposing that 5-HT levels are strongly dependent on a serotonin monitoring factor in the extracellular space that adjusts 5-HT release accordingly, while taking into account the impact of mechanisms for 5-HT clearance, namely the reuptake by the serotonin transporter and the degradation by metabolic enzymes. The model proposes that autistic individuals will have a molecular defect that impairs the feedback mechanism regulating serotonin release according to 5-HT levels, with the transporter and other molecules involved in serotonin clearance acting as modifiers. The evidence gathered in the present study indicates that the β3 integrin subunit may be involved in the regulation of serotonin release. It is known that αIIbβ3 integrin plays a key role in the regulation of platelet aggregation and serotonin secretion, through an outside–in signaling mechanism that is regulated by the Leu33Pro *ITGB3* polymorphism (Sajid et al. 2002; Vijayan et al. 2005). While the existence of the β3 integrin subunit in the EC cells model has not been documented, and thus no simple projection/extrapolation can be made, the role of *ITGB3* in platelets and its expression in the brain, where β3 integrins mediate functional pre and postsynaptic maturation of synapses, as well as evidence that blocking integrin activity after spinal cord injury improves serotonergic innervation (Oatway et al. 2005), support this hypothesis.

In summary, we present evidence for a common genetic mechanism underlying autism and serotonin level distribution, thus providing a molecular explanation for the association of platelet hyperserotonemia with autism and suggesting a plausible pathological mechanism. It is worth remarking that the alterations in platelet 5-HT levels do not necessarily imply changes in brain serotonin levels, but do reflect the dysfunction of brain expressed genes with consequences in brain development and function that may lead to autism symptomatology. Functional studies are now required to assess the biological meaning of the statistical interactions identified in this work, but the findings are compatible with biological observations and theoretical models, and thus provide a solid basis for functional work. Autism is a highly complex disorder, and testing for gene epistasis in association with specific disease associated phenotypes is shown to be a powerful strategy towards the identification of autism pathophysiology.

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