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Molecular Genetics of the Platelet Serotonin System in First-Degree Relatives of Patients with Autism

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Elevated platelet serotonin (5-hydroxytryptamine, 5-HT) is found in a subset of children with autism and in some of their first-degree relatives. Indices of the platelet serotonin system, including whole blood 5-HT, 5-HT binding affinity for the serotonin transporter (K_m), 5-HT uptake (V_{max}), and lysergic acid diethylamide (LSD) receptor binding, were previously studied in 24 first-degree relatives of probands with autism, half of whom were selected for elevated whole blood 5-HT levels. All subjects were then genotyped for selected polymorphisms at the *SLC6A4*, *HTR7*, *HTR2A*, *ITGB3*, and *TPH1* loci. Previous studies allowed an *a priori* prediction of *SLC6A4* haplotypes that separated the subjects into three groups that showed significantly different 5-HT binding affinity (K_m, $p = 0.005$) and 5-HT uptake rate (V_{max}, $p = 0.046$). Genotypes at four individual polymorphisms in *SLC6A4* were not associated with platelet 5-HT indices. Haplotypes at *SLC6A4* and individual genotypes of polymorphisms at *SLC6A4*, *HTR7*, *HTR2A*, *ITGB3*, and *TPH1* showed no significant association with whole blood 5-HT. Haplotype analysis of two polymorphisms in *TPH1* revealed a nominally significant association with whole blood 5-HT ($p = 0.046$). These initial studies of indices of the 5-HT system with several single-nucleotide polymorphisms at loci in this system generate hypotheses for testing in other samples.
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INTRODUCTION

Twin studies suggest that autism has a complex genetic etiology (Folstein and Rutter, 1977; Bailey *et al*, 1995). One clue into the pathophysiology of autism is elevated whole blood serotonin (5-hydroxytryptamine, 5-HT), present in approximately one-third of patients (Schain and Freedman, 1961; Cook and Leventhal, 1996). Hyperserotonemia has been found to be familial and to be associated with recurrence risk of autism within families (Abramson *et al*, 1989; Cook *et al*, 1990; Piven *et al*, 1991). Thus, genetic variation in the 5-HT pathway is likely to shed light on the genetic liability to the syndrome.

In our previous study of first-degree relatives of autism probands, whole blood 5-HT levels were found to correlate with 5-HT transport uptake V_{max}. Additionally, the hyperserotonemic subgroup was found to have decreased [^{3}H]-lysergic acid diethylamide (LSD)-labeled receptor binding. These two findings clustered in distinct subgroups. Of the eight hyperserotonemic subjects who had both 5-HT uptake and LSD binding studies performed, four subjects had 5-HT uptake that was at least 1 SD above the highest normoserotonemic subject and three different subjects had LSD binding that was at least 0.95 SDs below the lowest normoserotonemic subject (Cook *et al*, 1993).

The correlation between 5-HT transport and whole blood 5-HT could arise from variation in the gene encoding the 5-HT transporter (SERT, *SLC6A4*). Linkage and association studies have implicated *SLC6A4* in autism (Yonan *et al*, 2003; Stone *et al*, 2004; Devlin *et al*, 2005; Sutcliffe *et al*, 2005). The *SLC6A4* promoter polymorphism HTTLPR long–long (L/L) genotype is associated with increased 5-HT uptake into the platelet (Greenberg *et al*, 1999; Anderson *et al*, 2002). A functional polymorphism in intron 2 was

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associated with whole blood 5-HT levels in one study (Coutinho *et al*, 2004).

Recent studies suggest that other *SLC6A4* polymorphisms may also be important in conferring risk for autism. Kim *et al* (2002) described two single-nucleotide polymorphisms (SNPs) (SNP10 [rs2020936], and SNP11 [rs2020937]) that were strongly associated with autism. A haplotype defined by T alleles at these two SNPs (SNP10-SNP11 TT) was most highly associated with autism (Cook EH, unpublished data). In a mutation screen of *SLC6A4*, Sutcliffe *et al* (2005) found rare functional SERT amino-acid changes that lead to increased 5-HT transport in subjects with autism (Prasad *et al*, 2005). Another amino-acid variant that leads to increased SERT function, Ile425Val, has been detected in subjects with obsessive–compulsive disorder (Ozaki *et al*, 2003; Delorme *et al*, 2005).

In our original study of first-degree relatives of patients with autism, hyperserotonemic subjects were found to have decreased ^{3}H-LSD-marked receptor density (Cook *et al*, 1993). LSD binds to 5-HT receptors including 5-HT_{2A} and 5-HT_7, both of which are expressed in the platelet (Cook *et al*, 1994) (Cook EH, unpublished observation). Murphy *et al* (2006) recently described decreased cortical 5-HT_{2A} binding in Asperger's syndrome, supporting the possible relevance of the platelet model. A functional amino-acid variant and a possibly functional promoter SNP have been described in the 5-HT_{2A} gene (*HTR2A*) (Ozaki *et al*, 1997; Spurlock *et al*, 1998). One study found association at three adjacent polymorphisms in the first intron of *HTR7* in autism (Prathikanti S and Cook EH, unpublished data).

Additional proteins may also be important in modulating the 5-HT system within the platelet. Variation in the integrin $\beta 3$ gene (*ITGB3*) has been associated with platelet 5-HT in multiple populations, including probands with autism (Weiss *et al*, 2004, 2005b, 2006). Tryptophan hydroxylase 1 (*TPH1*), which catalyzes the rate-limiting step in 5-HT synthesis in the periphery, could also contain functional variation that would impact 5-HT synthesis.

Based on the platelet 5-HT indices in the two subpopulations of subjects with elevated platelet 5-HT, a series of hypotheses were generated. Polymorphisms and haplotypes in *SLC6A4* were hypothesized to be associated with variation in V_{max}, K_m, and whole blood 5-HT levels. Polymorphisms in *HTR7* and *HTR2A* were hypothesized to be associated with variation in LSD-binding and whole blood 5-HT levels. Finally, polymorphisms in *ITGB3* and *TPH1* were hypothesized to be associated with whole blood 5-HT levels. Our hypotheses were tested by genotyping the subjects previously studied for indices of the platelet serotonin system (Cook *et al*, 1993). Polymorphisms within each gene were selected based upon either reported association with autism or reported alteration in gene or protein function. In the case of *TPH1*, no known association with autism or functional variation has been reported, and two SNPs were chosen based upon position within a large haplotype block spanning the gene (Table 1).

METHODS

Subjects and Blood Collection

Subjects were selected as part of a previous study that measured indices of the platelet serotonin system (Cook *et al*, 1993). Two groups (normoserotonemic and hyperserotonemic) of 12 sex- and age-matched first-degree relatives of children with autism were studied (Table 2). Subjects were excluded if they had used psychotropic medication within 6 months of the study. Hyperserotonemia was defined as greater than 2 SDs above the mean as determined previously (Cook *et al*, 1988, 1993). Subjects were chosen

Table 2 Sample Information

Demographic and data variables	Hyperserotonemic subjects	Normoserotonemic subjects
Age years	38.1	38.2
Males	4	4
Females	8	8
Fathers of proband with autism	4	4
Mothers of probands with autism	7	7
Sisters of probands with autism	1	1
Caucasian	9	11
African-American	1	0
Hispanic	2	0
Asian	0	1

Table 1 HapMap Gene Coverage Data for Selected Single Nucleotide Polymorphisms

Polymorphism	Gene	HapMap haplotype block in March 2006 NCBI Build (Gabriel *et al*, 2002)	Haplotype block gene landmarks	Maximum span of $r^2 > 0.8$	$r^2 > 0.8$ gene landmarks
rs2020936/SNP10	*SLC6A4*	Chr 17: 25536104-25576899	Intron 1–3′ UTR	Chr 17: 25571336-25575014	Promoter - Intron 2
rs2020937/SNP11	*SLC6A4*	Chr 17: 25536104-25576899	Intron 1–3′ UTR	Data unavailable	
rs2185706	*HTR7*	Chr 10: 92564219-92643099	Flanking exon 1	Chr 10: 92592875-92614949	Flanking Exon 1
rs6311	*HTR2A*	Chr 13: 46367941-46373295	Promoter exon 1	Chr 13: 46369479-46372139	Promoter
rs6314	*HTR2A*	Chr 13: 46301361-46313002	Flanking exon 3	None	
rs5918	*ITGB3*	Not in haplotype block	N/A	Chr 17: 42691151-42725517	Intron 1–10
rs1799913	*TPH1*	Chr 11: 17955185-18023237	Flanking gene	Data unavailable	
rs623580	*TPH1*	Chr 11: 17955185-18023237	Flanking gene	None	

based on the samples collected in February 1992. Blood was drawn and all platelet serotonin indices described below were conducted as part of the original published study (Cook *et al*, 1993). Although whole blood 5-HT levels are typically stable within individuals, some of the hyperserotonemic subjects showed instability and were not in the hyperserotonemic range when sampled at the time of the platelet assays, which may be related to the seasonal variation that has been described previously (Wirz-Justice *et al*, 1977; Hanna *et al*, 1998). The whole blood 5-HT level from the day of the platelet studies was used for analyses in the present study.

Whole Blood 5-HT and Platelet Count

Whole blood 5-HT was chosen as the most reliable measure of platelet 5-HT because greater than 99% of whole blood 5-HT is in the platelet fraction (Anderson *et al*, 1987). Direct measurement of platelet 5-HT by centrifugation adds laboratory error due to 5-HT release during processing or variable platelet yield. Whole blood 5-HT was measured by HPLC with fluorometric detection (Anderson *et al*, 1981). Intra-assay and interassay coefficients of variation were 1.7 and 6.2%, respectively. Platelet count was determined with a Thrombocounter C (Coulter Electronics, Hialeah, FL). Time of sampling may affect whole blood 5-HT levels (Wirz-Justice *et al*, 1977); therefore, all of the blood sampling was carried out between 0900 and 1000 hours. Similarly, an influence of seasonal variation on whole blood 5-HT levels has been described (Wirz-Justice *et al*, 1977), but samples for these studies were collected exclusively in late summer and early fall.

Platelet 5-HT Uptake and [^{3}H]-LSD Binding

Platelet 5-HT uptake and [^{3}H]-LSD binding were assayed in the laboratory of Dr Ramesh Arora at Hines V.A. Hospital as described previously (Cook *et al*, 1993). Six samples could not be assayed for platelet 5-HT uptake for logistical reasons because the assay had to be performed on the same day as the blood draw. One sample did not have sufficient tissue for [^{3}H]LSD binding because of difficulty in blood drawing.

Platelet Count and Platelet 5-HT Uptake

Platelets were counted electronically by means of a Coulter thrombocounter in triplicate. 5-HT uptake was studied as described earlier (Arora and Meltzer, 1981). Briefly, platelet-rich plasma (0.3 ml) was preincubated with Kreb's phosphate buffer (0.6 ml, pH 7.4) minus $CaCl_2$ for 10 min at 37°C. Next 0.1 ml of [^{14}C]5-HT was added to yield final concentrations of 5-HT of 0.3, 0.5, 0.75, and 1 μM. One set of tubes was immediately immersed in an ice bath while the other set was incubated for an additional 4 min. Radioactivity in the platelet pellet was counted as described earlier (Arora and Meltzer, 1981).

[^{3}H]-LSD Binding

A 0.2 ml aliquot containing 100–150 μg of membrane protein was incubated with [^{3}H]-LSD (specific activity 79.9 Ci/mmol) in Tris HCl + NaCl + KCl buffer (pH 7.4) for 4 h at 37°C in the presence and absence of spiperone (300 nM). After incubation, the reaction was terminated by the addition of Tris-HCl (pH 7.7, 50 mM) containing 0.1% bovine serum albumin and rapidly filtered through GF/F filters. The filters were washed and the radioactivity was counted as described previously (Cook *et al*, 1993).

The specific binding of [^{3}H]-LSD was defined as the difference in binding in the presence and absence of spiperone. Five to six concentrations of [^{3}H]LSD (0.2–4 nM) were used in the incubation mixture to determine the dissociation constant (K_d) and the density of [^{3}H]-LSD biding sites (B_{max}) by Scatchard analysis.

DNA Isolation

DNA was isolated from 300 μl of frozen whole blood samples using the Puregene® DNA Purification method (Gentra Systems, Minneapolis, MN, http://www.gentra.com).

Genotyping

Single-Nucleotide Polymorphisms. Nine SNP markers were genotyped using the TaqMan® assay (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Six SNP markers were genotyped using the Assays-on-Demand™ with standard conditions (*HTR2A* rs6311/ C_8695278, *HTR7* rs2185706/ C_11756741, *HTR7* C_416286, *HTR7* C_8418463, *TPH1* rs1799913/C_2645661, and *TPH1* rs623580/C_2645676; whereas four SNP markers were genotyped using TaqMan® Assays-by-Design (*HTR2A* rs6314, *SLC6A4*-Ile425Val, *SLC6A4*-Gly56Ala, *SLC6A4*

Table 3 TaqMan® Assays-By-Design Primers

Polymorphism	Primer sequences	Probe sequences
HTR2A	Sense: 5′-ACTCCGTCGCTATTGTCTTTAGAAG-3′	
rs6314	Antisense: 5′-AGCAAGATGCCAAGACAACAGATAA-3′	
SLC6A4	Sense: 5′-GCAGAAGCGATAGCCAACATG-3′	VIC- 5′-CTTTCTTTGCCGTCATCT-3′
Ile425Val	Antisense: 5′-CAAGCCCAGCGTGATTAACATC-3′	FAM- 5′-TTTCTTTGCCATCATCT-3′
SLC6A4	Sense: 5′-GGGTACTCAGCAGTTCCAAGTC-3′	VIC-5′-CTGGTGCGGGAGAT-3′
Gly56Ala	Antisense: 5′-GGGATAGAGTGCCGTGTGT-3′	FAM-5′-CTGGTGCGGCAGAT-3′
SLC6A4	Sense: 5′-GCAAACACCACTCAGAAGGATATGA-3′	
rs2020936	Antisense: 5′-GGTGAGCAGGGTGAGGTT-3′	

SNP10/rs2020936). A control sample known to contain the *SLC6A4* Ile425Val variant (Glatt *et al*, 2001) from the DNA Polymorphism Discovery Resource (DPDR, http://locus.umdnj.edu/nigms/pdr.html) was used to confirm assay performance. Samples known to contain the *SLC6A4*-Gly56Ala variant were also genotyped to confirm assay performance (Sutcliffe *et al*, 2005). One subject was not genotyped for the two *TPH1* SNPs due to scarcity of DNA. Primers and probes for Assays-By-Design are shown in Table 3.

One SNP marker (*SLC6A4* SNP11/rs2020937) was genotyped by Fluorescence Polarization-Template Directed dye-terminator Incorporation assay (FP-TDI) (Chen *et al*, 1999) as described previously (Kim *et al*, 2002).

The *ITGB3* Leu33Pro SNP (rs5918) was genotyped with a restriction fragment length polymorphism assay using *Msp*I (New England Biolabs, http://www.neb.com) as described previously (Weiss *et al*, 2006).

Length Polymorphisms. The *SLC6A4* HTTLPR and intron 2 VNTR polymorphisms were amplified and detected as described previously (Kim *et al*, 2002).

Statistical Analysis

Haplotypes at the four common *SLC6A4* polymorphisms were predicted using web-based PHASE version 2.0. (Stephens *et al*, 2001; Stephens and Donnelly, 2003). Five haplotypes were predicted (Table 4). These were condensed to three groups of diplotypes using *a priori* predictions from analyses in other populations. Group 1 was homozygous for haplotypes containing the T allele at both *SLC6A4* SNP10 and SNP11. The SNP10-SNP11 TT haplotype was most highly associated with autism in a dense association mapping study (Cook EH, unpublished analysis of data from Kim *et al* (2002)), and we reasoned that it may be separately associated with altered transporter function and elevated whole blood 5-HT. Group 2 was homozygous for haplotypes containing the long allele of the HTTLPR polymorphism previously associated with elevated platelet 5-HT uptake (Greenberg *et al*, 1999; Anderson *et al*, 2002). Group 3 had other combinations of haplotypes. These three groups were compared by ANOVA for platelet 5-HT maximum uptake rate (V_{max}), platelet 5-HT affinity (K_m), and whole blood 5-HT levels. When significant association was detected, *post hoc* analysis by ANOVA compared three groups: group 1 consisted of subjects homozygous for haplotypes containing the T allele at both SNP10 and SNP11, group 2 consisted of subjects heterozygous for haplotypes containing the T allele at these two SNPs, and group 3 consisted of subjects without haplotypes containing the T allele at these two SNPs. Follow-up *t*-test was used to compare subjects homozygous for haplotypes containing the T allele at both SNP10 and SNP11 and all other subjects. When significant association was observed, follow-up analysis was performed excluding the four non-Caucasian subjects to remove potential population stratification bias. *Post hoc* analysis by ANOVA was also conducted to compare the three genotype groups for each individual *SLC6A4* marker.

Genotypes at each *HTR2A* and *HTR7* polymorphism were assessed for association with [^{3}H]-LSD binding and whole blood 5-HT using ANOVA and independent sample *t*-test. The *HTR2A* His452Tyr variant was only observed in three subjects, and haplotype analysis was not performed for the two *HTR2A* SNPs. The three *HTR7* SNPs (C_416286, C_8418436, and rs2185706) were found to be in perfect linkage disequilibrium (LD). Results reported for rs2185706, therefore, also apply to the other two *HTR7* SNPs. Haplotypes at the two *TPH1* SNPs were constructed by using web-based PHASE version 2.0 (Table 5) (Stephens *et al*, 2001; Stephens and Donnelly, 2003). Allele-wise haplotype analysis by ANOVA was used to compare whole blood 5-HT across the three observed haplotypes. Genotype at both *TPH1* SNPs was assessed for association with whole blood 5-HT using independent sample *t*-test. When significant association between *TPH1* haplotypes and whole blood 5-HT was detected, follow-up analyses were performed to examine potential sources of bias. To correct for possible effects of platelet count, three subjects with outlier platelet count values (below 150 000 or above 450 000 platelets per microliter) were eliminated from the analysis of *TPH1* haplotype effects and whole blood 5-HT per platelet was used as the dependent measure. To correct for possible effects of ethnicity, the four non-Caucasian subjects were eliminated from the analysis. Allele-wise haplotype analysis by ANOVA was used to compare whole blood 5-HT across the three observed haplotypes in the remaining 17 subjects.

Association at individual SNPs was assessed by ANOVA, or by *t*-test when only two genotype groups were present (rs6314) or when there were fewer than five subjects with a particular genotype (rs2185706, rs623580, and rs1799913). In the case of a homozygous genotype occurring fewer than five times, subjects were lumped with heterozygotes for statistical analysis. Bonferroni correction was not applied to these exploratory data. The Kolmogorov–Smirnov test was used to verify that each dependent variable did not deviate from normality.

Table 4 *SLC6A4* Haplotypes with Frequencies

Haplotype (HTTLPR–SNP10–SNP11–VNTR)	Number of occurrences
Short–T–T–12-copy	17
Short–C–T–12-copy	3
Long–T–T–12-copy	4
Long–C–T–12-copy	7
Long–T–A–9/10-copy	17

Table 5 *TPH1* Haplotypes with Frequencies

Haplotype (rs1799913–rs623580)	Number of occurrences*
G–T	10
T–T	21
G–A	15

*DNA was no longer available for one subject.

RESULTS

Platelet 5-HT Uptake V_{max} and K_m

ANOVA comparing SNP10-SNP11 TT/TT homozygotes, HTTLPR L/L homozygotes, and subjects with other haplotype combinations, revealed a significant effect on K_m ($F = 7.75$, $p = 0.005$, $n = 18$) and V_{max} ($F = 3.82$, $p = 0.046$, $n = 18$) across the three groups (see Figure 1). Follow-up analysis excluding the four non-Caucasian subjects confirmed a significant effect on K_m ($F = 5.770$, $p = 0.018$, $n = 14$) but did not show a significant effect on V_{max} ($F = 2.539$, $p = 0.120$, $n = 14$). Of note, all of the subjects in the SNP10-SNP11 TT/TT homozygote group had HTTLPR S/S or S/L genotypes. *Post hoc* ANOVA comparing SNP10-SNP11 TT/TT homozygotes, SNP10-SNP11 TT heterozygotes, and subjects with other haplotypes revealed a significant effect on K_m ($F = 6.29$, $p = 0.01$) but not on V_{max} ($F = 2.726$, $p = 0.098$). Follow-up analysis excluding the four non-Caucasian subjects confirmed a significant effect on K_m ($F = 4.315$, $p = 0.039$) but again not on V_{max} ($F = 2.217$, $p = 0.152$). *T*-test demonstrated that SNP10-SNP11 TT/TT homozygotes differed significantly from other subjects for K_m ($p = 0.002$) and V_{max} ($p = 0.029$). Follow-up analysis excluding the four non-Caucasian subjects confirmed a significant effect on K_m ($p = 0.009$) but revealed only a supportive trend for V_{max} ($p = 0.053$). ANOVAs for genotypes of each individual marker, including the HTTLPR, revealed no significant effects.

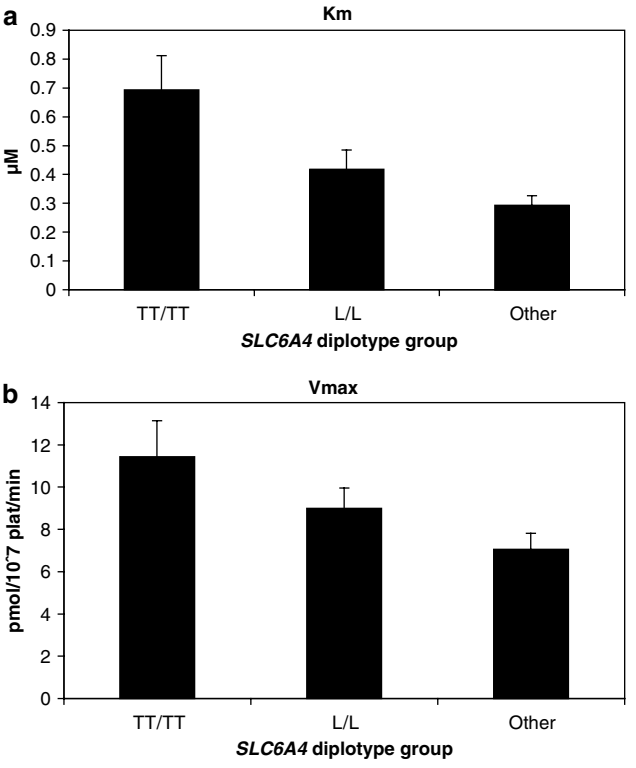

Figure 1 Altered K_m and V_{max} by *SLC6A4* diplotype group. Mean values with SEM of (a) [^{14}C]-5-HT binding affinity K_m and (b) [^{14}C]-5-HT uptake V_{max} are shown for each of three *SLC6A4* diplotype groups. Subjects in the TT/TT group had two haplotypes (ie a diplotype) containing the SNP10–SNP11 T–T haplotype. All of these subjects had HTTLPR S/S or S/L genotypes. Subjects in the L/L group had two haplotypes containing the HTTLPR L allele. Subjects in the 'other' group had other combinations of haplotypes and all had HTTLPR S/S or S/L genotypes.

[^{3}H]LSD Binding

Genotypes at the *HTR7* LD block and the two *HTR2A* polymorphisms were analyzed by ANOVA (rs6311) or independent sample *t*-test (rs6314 and rs2185706) for association with [^{3}H]-LSD binding (B_{max}). Although neither *HTR2A* polymorphism was found to be significantly associated with [^{3}H]LSD binding (rs6311 F-value = 1.431, $p = 0.263$ and rs6314 t-value = 0.736, $p = 0.470$, $n = 23$) a trend was observed for the *HTR7* SNP (rs2185706 t-value = 1.944, $p = 0.065$).

Whole Blood 5-HT

Analysis with PHASE generated three haplotypes between the two *TPH1* SNPs, indicating that these two SNPs are in complete, but not perfect, LD in this sample. Data from the HapMap project show a region of high LD in this region, including the entire *TPH1* gene ($D' = 0.92$–1.0; $r^2 = 0.10$–1.0; see Table 1) (The International HapMap Consortium, 2003). Allele-wise haplotype analysis by ANOVA revealed a significant effect on whole blood 5-HT ($F = 3.31$, $p = 0.046$, $n = 23$, see Figure 2). This effect remained significant after correction for platelet count and removal of non-Caucasian subjects and those with outlier platelet values ($F = 3.991$, $p = 0.029$, $n = 17$). Follow-up ANOVA applied to subjects with 0, 1, or 2 copies of the G–A haplotype revealed a significant effect ($F = 3.75$, $p = 0.041$). This effect also remained significant after correction for platelet count and removal of non-Caucasian subjects and those with outlier platelet values ($F = 6.267$, $p = 0.011$). Genotypes at the two individual *TPH1* polymorphisms were analyzed by independent sample *t*-test (rs623580 and rs1799913) for association with whole blood 5-HT, and a trend was observed for association with whole blood 5-HT at each SNP (rs623580, $p = 0.054$; rs1799913, $p = 0.059$). No significant effects of *SLC6A4* polymorphisms or haplotypes, or *HTR2A*, *HTR7*, or *ITGB3* genotypes on whole blood 5-HT were detected by ANOVA.

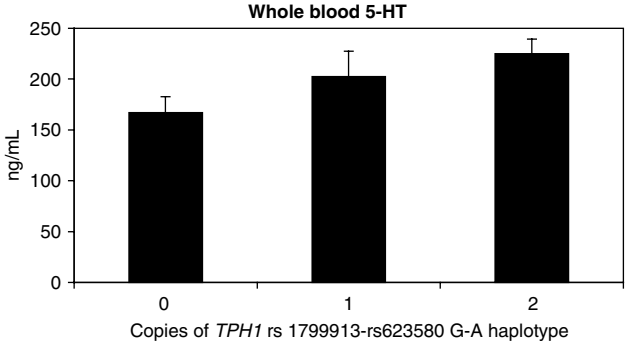

Figure 2 Altered whole blood 5-HT by *TPH1* diplotype group. Mean values with SEM of whole blood 5-HT are shown for subjects with three different *TPH1* diplotypes, containing either none, one, or two copies of the rs1799913–rs623580 haplotype.

Uncommon Functional SERT Variants

The Gly56Ala variant was detected in two normoserotonemic subjects and no hyperserotonemic subjects. No relationship was seen between this variant and whole blood 5-HT, K_m, or V_{max}. The Ile425Val variant was not present in our sample.

DISCUSSION

Hyperserotonemia occurs in approximately one-third of patients with autism (Cook and Leventhal, 1996) and is also familial, occurring in some first-degree relatives (Abramson *et al*, 1989; Cook *et al*, 1990). The association tests presented here are a continuing effort to understand these abnormalities in the 5-HT system. Unlike studies of candidate genes in the disorder itself, polymorphisms in candidate genes were studied in relationship to platelet 5-HT measures.

Significant differences were observed between different haplotype groups at *SLC6A4* for transporter K_m and V_{max}. Appropriate statistical correction for multiple testing is difficult to assess when analyzing multiple polymorphisms in LD with one another, but only the K_m association appears robust to correction. *Post hoc* analysis revealed that the difference in K_m was largely contained in the contrast between subjects homozygous for the SNP10-SNP11 TT haplotype and other subjects ($p = 0.002$). This is especially remarkable because these subjects all had HTTLPR S/S or S/L genotypes, which have been previously associated with lower 5-HT transport V_{max} in comparison with the L/L genotype (Greenberg *et al*, 1999; Anderson *et al*, 2002). This association suggests that the TT haplotype may be in LD with a functional variant or variants elsewhere in the gene or may itself have some unknown functional significance. The functional variant(s) could alter gene expression, protein trafficking, or protein regulation (Lesch *et al*, 1996; Prasad *et al*, 2005), and may demonstrate its platelet phenotype indirectly as a regulatory response to altered SERT structure or function (Jayanthi *et al*, 2005; Zhu *et al*, 2005; Carneiro and Blakely, 2006). The two most commonly observed amino-acid changes, Gly56Ala and Ile425Val, do not account for the association in this sample. Interestingly, although the SNP10-SNP11 TT haplotype is significantly associated with transporter function, it does not show a significant association with whole blood 5-HT itself.

The seeming contradiction of altered transporter function without altered platelet 5-HT levels points to a heterogeneous etiology of hyperserotonemia in autism, as predicted from the original study in which transporter V_{max} and LSD binding were elevated and decreased, respectively, in separate subgroups (Cook *et al*, 1993; Cook and Leventhal, 1996). Seasonal variation of platelet 5-HT, which has previously been described in association with the *SLC6A4* HTTLPR polymorphism (Hanna *et al*, 1998), could also add further complexity to the relationship between 5-HT levels and 5-HT transport function. It is possible that multiple polymorphisms at *SLC6A4* have varying effects on function that may interact with one another in ways that we cannot yet predict. These variants may include both common polymorphisms of small effect, such as HTTLPR or the intron 2 VNTR, as well as rare variants of larger effect, such as the Ile425Val variant. Our inability to detect a platelet phenotype in the two normoserotonemic subjects with the SERT Gly56Ala variant is not altogether surprising because this variant showed only one-third elevated 5-HT transport compared to the wild type in a cellular model (Prasad *et al*, 2005). A preponderance of other uncommon variation in the gene region suggests that other variants may also affect transcription or trafficking of *SLC6A4* (Sutcliffe *et al*, 2005). The genetic component of familial hyperserotonemia in autism also likely includes the effects of variants in other genes, whether tested here or still unknown.

Another independent contributor to familial hyperserotonemia in autism is altered 5-HT receptor binding. Two *HTR2A* polymorphisms showed no association with [^{3}H]LSD binding. Analysis of possible parent-of-origin effects was not possible for this variably imprinted gene (Kato *et al*, 1996, 1998; Bunzel *et al*, 1998). Polymorphisms in *HTR7* are also a potential source of altered [^{3}H]-LSD binding. A recent study found a nominal association between a set of three polymorphisms in strong LD with one another and autism (Prathikanti S and Cook EH, unpublished data). These polymorphisms were observed to be in perfect LD in our population and showed a trend toward association with [^{3}H]LSD binding.

Platelet hyperserotonemia could also be explained by variation in other proteins known to be important within the 5-HT system. Previous studies have found association between *ITGB3* and whole blood 5-HT levels in males (Weiss *et al*, 2004, 2005a, 2005b), but our study is too small to support a sex-specific analysis. Additionally, allelic heterogeneity with regard to 5-HT levels is evident at *ITGB3*. The amino-acid polymorphism that we genotyped in this study showed evidence for association with autism in previous studies, but did not show the strongest evidence for association with serotonin level in these studies (Weiss *et al*, 2005b, 2006). A haplotype of two *TPH1* polymorphisms was associated with whole blood 5-HT by allele-wise ANOVA ($p = 0.046$). These nominally significant findings would not survive statistical correction for multiple testing. If replicated, this association would suggest that *TPH1* variation may have important effects on platelet 5-HT synthesis in the periphery (Walther *et al*, 2003). These two SNPs are unlikely to be functional themselves, but high LD in this region suggests that they would be likely to be in LD with any common functional variation in this region (The International HapMap Consortium, 2003). Although it is not responsible for the majority of central 5-HT synthesis in the mature animal, *TPH1* remains a candidate for developmental disorders because of its role in 5-HT synthesis late in brain development (Nakamura *et al*, 2006).

The primary limitation of this study is sample size. We have limited power to detect association between genotype and indices of the platelet 5-HT system. A second limitation is the absence of family genotype data or genomic control in a quantitative genotype comparison study. Methodology has not yet been developed for genomic control when considering association with quantitative traits. Analysis including only Caucasian subjects supported the findings in the overall group of subjects.

These results build on previous research that found familial hyperserotonemia in autism to be associated with either increased 5-HT uptake or decreased receptor binding.

Variation in *SLC6A4* was associated with 5-HT binding and uptake but did not have a significant effect on whole blood 5-HT. Genotypes at *HTR7* polymorphisms previously associated with autism need further study in relation to [^{3}H]-LSD binding. Haplotypes at two polymorphisms in *TPH1* may be associated with whole blood 5-HT levels. The platelet hyperserotonemia story remains complex and will require additional study in a larger population. Complete characterization of the variation around the *SLC6A4* gene in a sample of patients or first-degree relatives with hyperserotonemia may be necessary to clarify the role of multiple potentially functional variants. Our data point to variation in other mechanisms that may play a larger role in hyperserotonemia than 5-HT uptake itself. Denser genotyping of polymorphisms and resequencing studies of *TPH1*, *ITGB3*, and *HTR7* may also be necessary to clarify their roles and identify functional variants that may be relevant to hyperserotonemia. Finally, a larger sample will enable analysis of possible allelic or gene–gene interaction within the peripheral serotonin system.

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