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ORIGINAL RESEARCH ARTICLE

Autism and the serotonin transporter: the long and short of it

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Autism is a neurodevelopmental disorder manifesting early in childhood. Some symptoms of autism are alleviated by treatment with selective serotonin reuptake inhibitors, which are known to interact with the serotonin transporter. Moreover, variation in the gene that encodes the transporter (*SLC6A4*), especially the HTTLPR locus, is known to modulate its expression. It is natural, therefore, to evaluate whether this variation plays a role in liability to autism. We investigated the impact of alleles at HTTLPR and three other loci in *SLC6A4* by using a large, independent family-based sample (390 families, 1528 individuals) from the NIH Collaborative Programs of Excellence in Autism (CPEA) network. Allele transmissions to individuals diagnosed with autism were biased only for HTTLPR, both for the narrow diagnosis of autism ($P = 0.035$) and for the broader diagnosis of autism spectrum ($P = 0.007$). The short allele of HTTLPR was significantly overtransmitted. Investigation of haplotype transmissions suggested that, in our data, biased transmission was only due to HTTLPR. With respect to this locus, there are now seven of 12 studies reporting significant transmission bias of HTTLPR alleles, a noteworthy result in itself. However, the studies with significant findings are almost equally divided between overtransmission of short and overtransmission of long alleles. We place our results within this extremely heterogeneous field of studies. Determining the factors influencing the relationship between autism phenotypes and HTTLPR variation, as well as other loci in *SLC6A4*, could be an important advance in our understanding of this complex disorder.
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Several studies implicate serotonin in the pathophysiology of autism (reviewed in Cook and Leventhal[1]). For individuals with autism, tryptophan depletion can worsen repetitive behaviors[2] and PET neuroimaging reveals evidence of impaired serotonin synthesis.[3] Furthermore, selective serotonin transporter inhibitors often reduce rituals and routines common to individuals with autism.[4] Hyperserotonemia is the most consistently replicated neurochemical finding in autism. A study comparing hyperserotonemic to normoserotonemic subjects suggests altered serotonin transporter function.[5] On the basis of these observations, Cook *et al*[6] evaluated alleles at the serotonin transporter gene (*SLC6A4*, MIM 182138) for linkage/association to autism. In the family-based association study of autism, they found the short allele of a variable number of tandem repeat (VNTR) polymorphism in the promoter region of *SLC6A4* was overtransmitted from parents to their autistic children. This locus is often identified as HTTLPR. By length it has two common alleles, short/long (S/L), and a small number of rare alleles. Their finding of linkage/association of HTTLPR and autism is even more intriguing when it is teamed with the observation that the S/L alleles have an impact on expression of *SLC6A4*.[7]

Unfortunately, since publication of the findings of Cook *et al*[6] results on this locus have been far from

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consistent. To our knowledge, of the studies examining transmission of S/L, three report significant overtransmission of S, three report significant overtransmission of L, and five report no evidence for significant, differential transmission (Table 1). Studies showing significant overtransmission of L have some of the smallest sample sizes and one study used a broader phenotype than autism and its spectrum disorders.[8]

Linkage studies yield equally ambiguous results. Older studies yield no strong evidence for—or against—linkage on chromosome 17q11.1–q12, the location of *SLC6A4*.[9–14] Three recent studies, on the other hand, yield much stronger support for a locus affecting liability to autism in the 17q11.1–q12 region: two studies targeted diagnosis *per se*;[15,16] the other used the presence/absence of rigid/compulsive features to show a strong signal for linkage in those families with compulsive behaviors and rigidity.[17]

By using a large number of families from the CPEA network, we examine linkage/association between liability to autism and four loci in *SLC6A4*: HTTLPR, the intron 2 VNTR polymorphism (also found to affect HTT expression), and two single nucleotide polymorphisms (SNPs) HTTSNP10 (RS2020936) and HTTSNP11 (RS2020937) in intron 1A.[18] We find evidence for linkage/association between liability to autism and the alleles of HTTLPR, but no evidence for linkage/association with alleles at other loci. We assess our results in light of the results from other studies.

Materials and methods

Simplex and multiplex autism families were recruited from ongoing research projects at six NIH CPEA Network sites: University of California Irvine, University of Pittsburgh, University of Rochester, University of Utah, University of Washington, and Yale University. Genotype data from 1528 individuals in 390 families were used for these analyses. Of these, approximately 37.7% were simplex families, 41% were multiplex families and the rest contained individuals who were not yet diagnosed. Of the multiplex families, most contained an affected sibling pair (97.5%), and a few were multigenerational (0.8%). With the exception of University of Rochester, all diagnoses were based on the Autism Diagnostic Interview-Revised (ADI-R),[19] the Autism Diagnostic Observation Schedule—Generic,[20] DSM-IV, and clinical evaluation to rule out known medical causes of autism. For the University of Rochester, individuals from 102 of the families were diagnosed as just described, and 95 families were diagnosed by clinical evaluation only. For most families, DNA was available from both parents, and 72.7% of all parents were genotyped. Based on the results of our previous studies of autism and other experience, most families were likely to be of European ancestry; nevertheless, only 37.5% of the families were known to be of European ancestry and 7% were known to be of ancestry other than European.

Genotypes for four *SLC6A4* loci were determined essentially as described by Kim *et al*.[18] Since the genotyping was performed at three different sites (Rochester, Washington, and Yale), a panel of 20 of the same control samples obtained from the Coriell Cell Repository was genotyped at each site and the results compared to insure that genotyping methods across sites produced identical results.

For our analyses, two groups of affected individuals were considered. The first group included all individuals diagnosed with autism, Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS), and Asperger's syndrome (broad diagnosis). The second group included only individuals diagnosed with autism (narrow diagnosis).

Markers and pedigrees were evaluated for single-locus Mendelian errors using the PedCheck program.[21] Mendelian transmission of haplotypes was evaluated by using eHap software[22] (http://wpicr.wpic.pitt.edu/WPICCompGen/), under the assumption that recombination did not occur among the *SLC6A4* loci in these pedigrees. Genotyping errors were set to missing. For the families analyzed herein, $<0.4\%$ of the genotypes were set to missing values. To test for differential transmission of alleles, FBAT was used (http://www.biostat.harvard.edu/~fbat/). FBAT implements a generalization[23] of the TDT test.[24] To analyze linkage/association, we chose an additive model, which is often powerful even when the true model deviates from additivity. Data were analyzed for both broad and narrow diagnosis. All analyses accounted for the possibility of linkage in the region surrounding *SLC6A4* by using FBAT's empirical test procedure. Haplotype-based analyses were performed using the software eHap.[22] The program Allegro[25] was used to perform two-point and multipoint linkage analyses.

To evaluate the power of the samples (Table 1) to detect excess transmission of S relative to L, we use the program PBAT.[26,27] For input, PBAT requires the family structures present in the sample, in terms of the number of genotyped affected and unaffected offspring and the number of genotyped parents. It also requires specification of the genetic model. For these calculations, we assume autism prevalence is $K=0.005$ and a targeted significance level of 0.05; the frequency of the disease allele equals the observed frequency of short alleles; specify the 'attributable fraction' $AF=1-f_0/K$,[26] in which f_0 is the probability of being affected with autism given the individual's genotype is homozygous L/L; and perform the calculations assuming a 4 : 3 transmission of S : L to autistic individuals. Please see Supplementary Information for how quantities such as the transmission ratio and AF inter-relate.

Results

To test whether alleles of loci in *SLC6A4* affect liability to autism, we genotyped four polymorphic sites (HTTLPR, intron 2 VNTR, HTTSNP10, and

Table 1 Summary of published studies of the transmission of HTTLPR alleles to autistic individuals

Study	*Sample*	*S*	*L*	*Ratio*	*Power*
Cook *et al*[6] +	86 trios	48	29	1.66	0.163
Conroy *et al*[39] +	84 trios	55	34	1.62	0.160
Kim *et al*[18]	81 trios	43	30	1.43	0.156
Betancur *et al*[32]	43 trios 53 multiplex	71	55	1.29	0.251
This study[a] +	103 trios 125 multiplex	175	137	1.28	0.447
McCauley *et al*[17] +	123 multiplex	221	180	1.23	0.382
Persico *et al*[40]	86 trios 5 multiplex	48	42	1.14	0.176
Coutinho *et al*[31]	182 trios	88	79	1.11	0.296
Maestrini *et al*[41]	8 trios 82 multiplex	72	76	0.94	0.284
Tordjman *et al*[38] −	69 trios	40	64	0.63	0.143
Klauck *et al*[8] −	65 trios	23	40	0.58	0.136
Yirmiya *et al*[42] −	34 trios	11	25	0.44	0.094

By a 'trio' we mean an affected individual and the two parents were genotyped; for multiplex, in addition to parents, more than one affected individual per family was genotyped (for purposes of calculating power, it is assumed that multiplex families have two affected offspring). Table entries ordered by 'ratio', the ratio of the number of transmitted S and L alleles. Studies marked with a '+' report significant overtransmission of S, those marked with a '−' report significant overtransmission of L. Power calculated as described in Materials and methods, Results, and Supplementary Information.
[a]We counted only the completely unambiguous transmissions of S and L, neglecting multigenerational pedigrees.

HTTSNP11) in a sample of 390 families. HTTLPR and the intron 2 VNTR were selected because these sites were both shown to be associated with autism in previous studies (Table 1), and both sites also appear to affect expression levels of *SLC6A4*. HTTSNP10 and HTTSNP11 were selected based on results from a comprehensive analysis of polymorphisms spanning the entire 38 kb *SLC6A4* gene.[18] In that study, 20 SNP sites were analyzed in 81 autism trios using TDT methods. Of the SNP's tested, alleles for HTTSNP9 and HTTSNP11 yielded the most significant evidence for association with autism. For these SNPs, however, an 'A' at HTTSNP11 is almost always teamed with a 'T' allele at HTTSNP9, and a 'T' at HTTSNP11 is almost always teamed with a 'C' allele at HTTSNP9. Since the information from the two loci is largely redundant, only HTTSNP11 was genotyped for this study. HTTSNP10 was selected because, when used to generate haplotypes with HTTSNP11, strong evidence for association was obtained (unpublished data, Edwin H Cook).

The allele distribution for each locus and our sample of pedigree founders, mostly parents of children with autism, was consistent with other studies (Table 2).[18] Founder genotypes conformed to Hardy–Weinberg expectations, with the exception of the HTTSNP10 (HTTLPR: $\chi^2 = 0.52$, DF = 1, $P = 0.47$; intron 2 VNTR: $\chi^2 = 2.53$, DF = 2, $P = 0.28$; HTTSNP11: $\chi^2 = 0.81$, DF = 1, $P = 0.37$; HTTSNP10: $\chi^2 = 6.49$, DF = 1, $P = 0.01$). There was an excess of both homozygotes at HTTSNP10.

Ignoring very rare alleles (Table 2), there were $2 \times 3 \times 2 \times 2 = 24$ possible haplotypes from these four loci. By using maximum likelihood estimation and the distribution of multilocus genotypes of parents and their offspring, we found evidence for 11 of these haplotypes in our sample (Table 3). Two were common, namely LTA10 and STT12, and account for roughly 55% of the chromosomes sampled. Paralleling the haplotype distribution, pairwise linkage disequilibrium (LD) among the four loci was substantial but never absolute (Table 3). Strongest LD was seen between the alleles at the Intron 2 VNTR and HTTSNP11 and least LD between alleles of HTTLPR and HTTSNP10.

For the broad diagnostic category, significant deviation from the null hypothesis of Mendelian transmissions was seen for the HTTLPR locus (Table 4), but none of the other loci. Similar results were obtained for the narrow diagnosis of autism, and conclusions were unchanged if the data were restricted to families of *known* European ancestry (Table 4). To ensure that the excess transmission of short alleles was restricted to autistic individuals, we

Table 2 Allele distributions for the *SLC6A4* loci

Locus (allele/relative frequency)			
HTTLPR	HTTSNP10	HTTSNP11	Intron 2 VNTR
S/0.407	C/0.201	A/0.388	9/0.013
L/0.593	T/0.799	T/0.612	10/0.376
ML/0.001			12/0.611
XL/0.001			

For HTTLPR, allele designations S and L are as previously described[43] and the ML and XL are rare alleles that are longer than the L allele. For HTTSNP10 and HTTSNP11, allele designations refer to the single nucleotide at the polymorphic site. Allele designations for the Intron 2 VNTR are as described previously.[6,44] The allele distributions are derived from parents' genotypes; sample sizes for the loci are 596, 591, 597 and 597 genotypes, respectively.

Table 3 Haplotype distribution and linkage disequilibrium statistics (absolute value of Lewontin's D′ (top) and r (bottom))

Haplotype	*Relative frequency*
LCT12	0.134
LTA9	0.011
LTA10	0.286
LTA12	0.006
LTT10	0.011
LTT12	0.146
SCT12	0.056
STA10	0.075
STA12	0.006
STT10	0.005
STT12	0.263

	HTTLPR	*HTTSNP10*	*HTTSNP11*	*Intron 2 VNTR*
HTTLPR	—	0.28	0.47	0.48
HTTSNP10	0.11	—	1.0	1.0
HTTSNP11	0.31	0.38	—	0.95
Intron 2 VNTR	0.32	0.38	0.94	—

Linkage disequilibrium statistics computed as if the HTTLPR and Intron 2 VNTR were biallelic for their common alleles. Haplotype designations are a list of alleles in the following order: HTTLPR, HTTSNP10, HTTSNP11, Intron 2 VNTR. Relative frequencies computed from all 390 families.

Table 4 Results of tests for biased transmission for four loci in *SLC6A4*, by sample

Locus	*Sample*			
	Broad	*Narrow*	*EA*[a] *ancestry*	*Unaffected*[b]
HTTLPR	9.80; 2; 0.007; 183	6.74; 2; 0.035; 102	5.03; 1; 0.025; 106	0.83; 2; 0.669; 56
HTTSNP10	0.00; 1; 0.947; 139	0.18; 1; 0.672; 74	0.03; 1; 0.862; 71	3.30; 1; 0.069; 40
HTTSNP11	0.15; 1; 0.701; 210	0.01; 1; 0.940; 111	0.24; 1; 0.624; 116	0.95; 1; 0.329; 61
Intron 2 VNTR	0.44; 2; 0.802; 206	0.02; 2; 0.989; 106	0.63; 2; 0.728; 114	1.64; 2; 0.441; 56.5

Values reported in each cell: χ^2 statistic; degrees-of-freedom; *P*-value; and effective number of informative transmissions.
[a]EA = European American, broad diagnosis.
[b]All children diagnosed as unaffected.

also analyzed transmission to unaffected offspring (127 unaffected offspring distributed in 108 families). No significant nonMendelian transmission was observed for any of the four loci (Table 4).

No significant nonMendelian transmissions were observed by analyzing the data as four-locus haplotypes ($\chi^2 = 14.09$; DF = 10; $P = 0.169$). However, haplotypes bearing the S allele and the 10 alleles at Intron 2 VNTR were notably overtransmitted ($\chi^2 = 6.29$; DF = 1; $P = 0.012$). To investigate whether the data suggest heterogeneity in the transmission of intron 2 alleles on the basis of the transmitted HTTLPR allele, we conducted a series of statistical contrasts of transmission rates by using eHap software.[22] We could find no significant evidence for differential transmission rates of the 10 *vs* 12 allele when they were transmitted along with an S allele ($\chi^2 = 0.04$; DF = 1; $P = 0.836$) or with an L allele ($\chi^2 = 0.21$; DF = 1; $P = 0.645$). Therefore, the haplotype analyses supported the single locus tests, suggesting HTTLPR alleles as the sole target of asymmetric transmission.

Despite evidence for linkage and association[28] in the form of excess transmission of S *vs* L, the multiplex families yielded no evidence for linkage. Two-point linkage analyses, conducted by using the estimated haplotype distribution (Table 3), and multipoint linkage analyses, conducted by treating the four loci as independent, produced indistinguishable results, NPL score ≈ -0.30 and ZLR score ≈ -0.31. When considering these results, it is important to bear in mind that the S allele is quite common in the population (Table 2). Therefore, even if S were to convey liability to autism, evidence for linkage would often be eroded by bilineal transmission of S alleles. Thus, *a priori*, we expected low power for the linkage test.

For the reported studies of HTTLPR (Table 1), analysis of the transmission rates of S : L revealed significant heterogeneity ($\chi^2 = 31.97$; DF = 11; $P = 0.0008$). This result was unsurprising given the set of findings, in which certain studies showed significant overtransmission of S and others show significant overtransmission of L. The source or sources of this heterogeneity remains unclear, and its presence argues against estimation of a common transmission rate. Even if we were to limit our calculations to studies in which transmission rates were greater than 1.0 (Table 1), however, the estimated transmission rate of S : L would not be large, 3.83 : 3 S : L. (This is the Mantel–Hanzel estimate; using all studies, the estimate naturally is lower, 3.4 : 3.) It is interesting to evaluate whether the samples were large enough to detect such a differential transmission rate of HTTLPR alleles. For this analysis, we assumed the frequency of the S allele was 0.41 (Table 2), and assumed a transmission ratio of 4 : 3 S : L to affected offspring. In fact, none of the studies had good power (>80%) to detect a 4 : 3 rate of overtransmission of S relative to L (Table 1).

Discussion

We evaluate four loci in *SLC6A4* to determine if these variants show linkage/association to diagnosis of autism. Of these loci, only the S/L alleles of HTTLPR show significant association. In our study, the S allele is significantly overtransmitted relative to the L allele. In terms of haplotypes, after accounting for the effect of S/L, no other locus showed nonMendelian transmission to autistic individuals.

Recently Stone *et al*[29] reported an enhanced signal for linkage in the 17q11.1–q12 region, which contains *SLC6A4*. The greater linkage signal occurs when they split their multiplex families into two groups according to whether the affected individuals within each family were only male (MO) or conversely whether the family contained at least one female who was affected with autism (FC). Substantially greater linkage occurs in the MO sample, which accounts for slightly more than one-half of the multiplex families. To determine if our results parallel those of Stone *et al*[29] we split our multiplex families according to the MO and FC criteria, and test for differential transmission. The data do not support differential transmission between the MO and FC families for any of the loci. For HTTLPR, both samples show marginally significant overtransmission of S (MO, $P = 0.057$; FC, $P = 0.090$), which is due to larger sample size of MO (99 families) *vs* FC (53 families) sample rather than different transmission rates (MO, 1.18; FC, 1.19).

Among the studies published to date, the significant heterogeneity of S *vs* L transmission stands out. Especially curious is the number of studies showing biased transmission of alleles, irrespective of what allele shows the bias. In fact, seven of 12 studies yield significant transmission distortion (Table 1), a rate that is itself significantly different from that expected under the null (seven out of 12 *vs* 0.6 out of 12 expected by chance (ie, $0.05 \times 12 = 0.6$)). This observation could be misleading due to the possibility of publication bias, if studies without significant results were unlikely to be published. Clearly, such a bias cannot be ruled out. On the other hand, it seems unlikely to be the sole explanation, given the controversial nature of the association of this locus with autism and phenotypes related to autism.

It is conceivable that this heterogeneity maps onto the well-known clinical and/or phenotypic heterogeneity of autism and its spectrum disorders. Under such a scenario, transmission of L would convey a greater likelihood of certain autism-related phenotypes compared to transmission of S, and *vice versa*. Notably, on the basis of serotonin blood levels being above or below the population mean, Persico *et al*[30] find excess inheritance of L when autistic individuals have above-average serotonin levels and excess inheritance of the S allele otherwise. Likewise, Coutinho *et al*[31] find a relationship between S/L alleles and serotonin blood levels. Yet, according to their findings, the S/L variant explains only a small portion of the variation in serotonin blood levels of autistic patients (see also Betancur *et al*[32]).

With respect to behavior, anxiety disorder, major depression, and obsessive-compulsive disorder are all elevated in first-degree relatives of probands with autism.[33–35] Many of these behaviors have been studied for their association with HTTLPR alleles. Although review of these association findings is beyond the scope of this discussion, it is of note that positive findings in depression and anxiety disorders[36] show overtransmission of the short HTTLPR allele, while a positive finding in OCD showed overtransmission of the long allele.[37]

The hypothesis that alleles at HTTLPR might alter the phenotypic expression of autism, as opposed to the liability to autism itself, was first advanced by Tordjman *et al*.[38] From the data in Table 1, the hypothesis seems quite plausible. On the other hand, it is also possible that the S allele, or another allele in tight LD with it, confers liability to autism. If this were the case, it could be a rather important locus due to high frequency of the S allele. Suppose, for example, that the ratio of transmission of S : L was 4 : 3, as we supposed for some of our analyses. Then the HTTLPR locus would account for a substantial portion of the population attributable risk, roughly 18% of the liability. Even if the ratio were 6 : 5, it would still account for roughly 11% of the liability. As our power calculations demonstrate, however, substantial samples are required to ensure detection for either of these differential transmission rates, larger than any study has thus far amassed. Resolution of the impact of variation in HTTLPR on liability to autism or phenotypes related to autism will be challenging but could be key to our understanding of the etiology of autism.

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Supplementary Information accompanies the paper on Molecular Psychiatry website (http://www.nature.com/mp).