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Allelic Heterogeneity at the Serotonin Transporter Locus (*SLC6A4*) Confers Susceptibility to Autism and Rigid-Compulsive Behaviors

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Autism is a spectrum of neurodevelopmental disorders with a primarily genetic etiology exhibiting deficits in (1) development of language and (2) social relationships and (3) patterns of repetitive, restricted behaviors or interests and resistance to change. Elevated platelet serotonin (5-HT) in 20%–25% of cases and efficacy of selective 5-HT reuptake inhibitors (SSRIs) in treating anxiety, depression, and repetitive behaviors points to the 5-HT transporter (5-HTT; SERT) as a strong candidate gene. Association studies involving the functional insertion/deletion polymorphism in the promoter (5-HTTLPR) and a polymorphism in intron 2 are inconclusive, possibly because of phenotypic heterogeneity. Nonetheless, mounting evidence for genetic linkage of autism to the chromosome 17q11.2 region that harbors the SERT locus (*SLC6A4*) supports a genetic effect at or near this gene. We confirm recent reports of sex-biased genetic effects in 17q by showing highly significant linkage driven by families with only affected males. Association with common alleles fails to explain observed linkage; therefore, we hypothesized that preferential transmission of multiple alleles does explain it. From 120 families, most contributing to linkage at 17q11.2, we found four coding substitutions at highly conserved positions and 15 other variants in 5′ noncoding and other intronic regions transmitted in families exhibiting increased rigid-compulsive behaviors. In the aggregate, these variants show significant linkage to and association with autism. Our data provide strong support for a collection of multiple, often rare, alleles at *SLC6A4* as imposing risk of autism.

Introduction

In 1943, Leo Kanner published a series of 11 case reports of children with a condition he termed "infantile autism" or "autistic disturbances of affective contact" (Kanner 1943). Autism (MIM 209850) is now recognized as a spectrum of phenotypes that spans a range of clinical severity but fundamentally represents deficits in three domains: (1) development and use of language, (2) development of social relationships and interactions with family and peers, and (3) patterns of repetitive behaviors, restricted interests and activities, and a strong desire to maintain "sameness" in environment and daily routines (reviewed by Folstein and Rosen-Sheidley [2001]). Pharmacotherapies include antipsychotic medications for disruptive and aggressive behaviors and selective serotonin-reuptake inhibitors (SSRIs) for treatment of anxiety, depression, and repetitive behaviors (Cook and Leventhal 1996; Hollander et al. 2005). Of interest regarding the partial efficacy of SSRIs are findings of elevated levels of platelet serotonin (5-hydroxytryptamine [5-HT]) in ~20%–25% of affected individuals (Schain and Freedman 1961) and correlation with levels in first-degree relatives (Cook et al. 1993). Other findings supporting 5-HT involvement in autism are reviewed elsewhere (Cook and Leventhal 1996; Veenstra-VanderWeele et al. 2000).

Since the first twin study of autism by Folstein and Rutter in 1977 (Folstein and Rutter 1977), evidence has mounted to support a predominantly genetic etiology of autism (reviewed by Folstein and Rosen-Sheidley [2001]). The prevalence of narrowly defined autism is ~1/1,000 (Chakrabarti and Fombonne 2001), and inclusion of the broader spectrum increases this rate to ~1/300–1/500 (Fombonne 2003; Yeargin-Allsopp et al. 2003). Males are affected more often, with a male:female ratio of 4:1. Twin data show that MZ twins have an average concordance of ~60%–70% for classic autism and up to 90% when milder language and social deficits seen in the broader phenotype are considered. This contrasts with DZ twin concordance rates, shown to be 0%–10%, depending on the study. Sibling-recurrence risk in narrowly defined autism is ~6%–8% (Jones and Szatmari 1988; Ritvo et al. 1989). Modeling the above data

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Table 1

Autism Family Samples for Linkage and Association Studies

	No. of Families						
	Multiplex			Trio			Grand
Sample	MO	FC	Total	MO	FC	Total	Total
AGRE	156	111	267	11	5	16	283
Tufts/Vanderbilt University	46	28	74	22	5	27	101
Total	202	139	341	33	10	43	384

has led to estimates of ~5–15 genes that contribute to genetic risk, possibly involving epistasis (Pickles et al. 1995), and locus heterogeneity (Risch et al. 1999). The data may best be explained by oligogenic inheritance, with different families possessing varying constellations of risk alleles (Folstein and Rosen-Sheidley 2001). A genetic-heterogeneity framework has important implications for the clinical variability observed in autism, in that specific risk loci, or distinct alleles at a given locus, are likely to influence the phenotype differently.

Several groups have undertaken family-based genetic studies to (1) identify regions of the genome commonly inherited by affected family members in multiplex-family samples (i.e., genomic linkage screens) and/or (2) test-specific loci for evidence of common alleles that confer genetic risk on the basis of tests of allelic association (reviewed by Folstein and Rosen-Sheidley [2001] and Veenstra-VanderWeele et al. [2004]). Chromosomal intervals identified in linkage studies with use of either a categorical diagnosis or indexing on specific traits include 7q, 2q, and 17q (International Molecular Genetic Study of Autism Consortium [IMGSAC] 1998, 2001*a*, 2001*b*; Ashley-Koch et al. 1999; Collaborative Linkage Study of Autism (CLSA) et al. 1999; Philippe et al. 1999; Risch et al. 1999; Auranen et al. 2000; Buxbaum et al. 2001, 2004; CLSA 2001; Liu et al. 2001; Shao et al. 2002*a*, 2002*b*; Yonan et al. 2003; McCauley et al. 2004, 2005; Stone et al. 2004; Cantor et al. 2005). For 17q, second-stage genomic screens with use of different samples (IMGSAC 2001*a*; Yonan et al. 2003) detected highly suggestive or significant linkage at or near the serotonin transporter (SERT) locus (*SLC6A4* [MIM 182138]) and evidence of a sex-restricted pattern of genetic effects (Stone et al. 2004). Our own reports of linkage to this region, with use of family data sets that contain partial overlap with the Autism Genetics Resource Exchange (AGRE) sample (Yonan et al. 2003), lend further support to involvement of this region (McCauley et al. 2004, 2005).

Studies of allelic association at *SLC6A4* with autism have focused primarily on a functional, insertion-deletion polymorphism in the promoter (5-HTTLPR) or a variable number tandem repeat (VNTR) marker in intron 2. Results of these studies are inconsistent, with associations shown either to the short (S) or long (L) alleles or absent association (Cook et al. 1997; Klauck et al. 1997; Maestrini et al. 1999; Persico et al. 2000; Tordjman et al. 2001; Yirmiya et al. 2001). Four studies have reported more-comprehensive analyses of common alleles and haplotypes that span the locus, including multiple SNPs (Kim et al. 2002; Conroy et al. 2004; McCauley et al. 2004; Devlin et al., in press). These studies find at least nominal association of the S allele at 5-HTTLPR and other markers or haplotypes. Our study of multiplex families (McCauley et al. 2004) also showed very suggestive linkage that cannot be explained by the modest association of HTTLPR and *rs140700* at *SLC6A4*. The two possible explanations for these results are (1) that *SLC6A4* is not the risk locus accounting for linkage or (2) that multiple different alleles at *SLC6A4* contribute to genetic risk independently. We report further investigation of chromosome 17 linkage in families with autism and an in-depth analysis of the *SLC6A4* gene, testing the hypothesis that allelic heterogeneity may account for the genetic liability to autism. Using a large sample of multiplex families, we find that *SLC6A4* exhibits strong evidence of linkage to autism, driven by allele-sharing in males. We find multiple coding and noncoding variants preferentially transmitted to affected individuals, and we identify significant correlations with increased rigid-compulsive behaviors, which indicates that *SLC6A4* is a likely susceptibility locus for autism, where allelic heterogeneity supports disease risk.

Subjects and Methods

Sample and Genetic Analyses

The sample for the present study consisted of 73 families recruited from the Tufts-Vanderbilt Consortium and 267 from the AGRE Consortium (table 1). The demographics of these populations have been reported elsewhere (Yonan et al. 2003; McCauley et al. 2005) as have diagnostic inclusion and exclusion criteria for linkage analysis (McCauley et al. 2005). Families were selected on the basis of the presence of a single proband who met full Autism Diagnostic Interview (ADI) criteria for autism and a second sibling who received the diagnosis

of autism or presented on the broader spectrum. In the current report, we further examined linkage in a larger sample of 341 families with at least two affected children; for association studies and screening for known variants (e.g., Gly56Ala), we included an additional 43 trios (proband and both parents). As reported elsewhere (McCauley et al. 2005), Tufts/Vanderbilt families were genotyped at deCODE by use of the deCODE 500 marker panel with an average intermarker spacing of ~8 cM. Two-point and multipoint heterogeneity LOD (HLOD) scores for individual and combined samples, respectively, were calculated with Allegro, under both dominant and recessive models (Gudbjartsson et al. 2000). Disease-allele frequencies were estimated to be 0.01 and 0.1 for dominant and recessive models, respectively. Phenotypic status was considered only for affected individuals, and other family members were designated as having an unknown phenotypic status. Nonparametric allele-sharing LOD* values were calculated using affected relative pair data that was based on an exponential model with use of the S_{pairs} scoring function, as recommended by McPeek (1999). Nonparametric linkage (NPL) scores and corresponding *P* values were also calculated with Allegro. The position of chromosome 17 markers is based on the deCODE genetic map (Kong et al. 2002).

Association tests of 5-HTTLPR and *rs140700* were performed using the pedigree disequilibrium test (PDT) statistic (Martin et al. 2000), a variant of the transmission/disequilibrium test (TDT), developed for use with general pedigrees. Genotype analysis of these markers has been reported elsewhere (McCauley et al. 2004). Other tests of association or comparison of allele frequencies involved generation of a χ^2 statistic and corresponding *P* values with use of standard 2×2 contingency tables. Comparison of male:female affection for autism in the presence of Gly56Ala alleles was performed using Fisher's exact test. Comparison of ADI-derived variable cluster scores was performed using the *T* test—with incorporation of subject numbers, means, and standard deviations for the overall data set and either (1) individuals within a given family or (2) family means across multiple families—to evaluate the significance of score differences. Significance is reported as two-tailed *P* values. Approval for these studies was granted by the respective institutional review boards at Tufts University School of Medicine/New England Medical Center and Vanderbilt University Medical Center. All studies were performed with the informed consent of the families participating in the research.

Variant Discovery

Known but rare *SLC6A4* coding variants reported in previous studies (Cargill et al. 1999; Glatt et al. 2001; Hahn and Blakely 2002; Ozaki et al. 2003) were screened in 327 multiplex families and 57 parent-child trios by use of TaqMan allelic discrimination assays (table 2). When possible, variants were assayed within exons to permit inclusion of in vitro mutagenized plasmid cDNA samples either alone or mixed with wild-type cDNAs, to provide homozygous and heterozygous controls, respectively. ABI TaqMan (Holloway et al. 1999) reactions were performed in a 5-μl volume in accordance with manufacturer's recommendations (Applied Biosystems). Cycling conditions included an initial denaturation at 95°C for 10 min, followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. Samples were analyzed using an ABI 7900HT Sequence Detection System.

Screening for unknown variants involved arbitrary selection of one affected individual from each of 120 families, ranked by family-specific nonparametric LOD* scores from the overall 341-family data set. Screening of PCR products for all exons was performed on the first 24 samples, with temperature-gradient capillary electrophoresis (TGCE) (Li et al. 2002), on a 96-capillary Reveal system, in accordance with manufacturer's recommendations (Spectrumedix). Putative variants detected by TGCE were confirmed by direct sequencing of PCR product by use of ABI dye terminators in the Center for Molecular Neuroscience Neurogenomics Core. The promoter for the initial 24 individuals and both exons and the promoter for samples from one proband from each of the remaining 96 (of 120) families were analyzed for variation by double-stranded direct sequencing of PCR products with use of ABI dye terminator chemistry. ABI electropherogram data obtained from Vanderbilt Cores or from Polymorphic DNA Technologies were imported and were analyzed for variation with the Phred/Phrap/Consed and PolyPhred suite of sequence analysis tools (Nickerson et al. 1997, 2001; Gordon et al. 2001). Variant confirmation and segregation of rare variants were determined by sequencing available family members and the original proband in the same manner. Location of variation within the gene was documented in table 3 with nomenclature described by den Dunnen and Antonarakis (2001).

Functional Analysis of Gly56Ala Activity and Regulation

Epstein-Barr virus (EBV)–transformed lymphocyte cell lines from AGRE or Tufts/Vanderbilt families with autism who carried either the Gly56 or 56Ala allele were obtained from the National Institute of Mental Health

Table 2

***SLC6A4* SNP Marker Information**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 3

TD of Multiple Coding and Noncoding Alleles at *SLC6A4*

Location and dbSNP Number	Position[a]	Protein	No. of Families	No. of Transmissions to		No. of NT	No. of Autistic	
				All Affected Individuals[b]	All but Proband[c]		Males	Females
Exon 1b:								
ss38318598	c.147C→A	5′ UTR	2	4	2	1	3	1
Exon 2:								
rs6355	c.462G→C	Gly56Ala[d]	7	15	8	5	11	4
Exon 9:								
ss38318599	c.1568A→C	Ile425Leu	1	2	1	0	2	0
Exon 10:								
ss38318600	c.1688T→C	Phe465Leu	1	2	1	1	1	1
Exon 12:								
ss38318601	c.1943G→C	Leu550Val	1	2	1	0	2	0
Exon 13:								
rs6352	c.2110A→C	Lys605Asn	1	0	0	2	0	0
Exon 14:								
rs13306796	c.2516A→G	3′ UTR	1	2	1	0	2	0
Promoter:								
ss38318589	g.15622G→A	NA	1	1	0	1	1	0
rs2020932	g.14519A→T	NA	3	8	5	0	6	0
ss38318590	g.14289A→C	NA	1[e]	1	0	1	1	0
rs25533	g.13912T→C	NA	1	4	3	0	2	0
ss38318591	g.13754C→T	NA	(1)[f]	(1)	(0)	(1)	(1)	(0)
Intron 1a:								
ss38318592	IVS1a+20C→T	NA	1	1	0	0	1	0
ss38318593	IVS1a+133G→A	NA	1	1	0	0	1	0
hcv11414117	IVS1a−47G→C	NA	5	10	5	2	8	2
hcv11414114	IVS1a−25G→A	NA	4	9	5	0	7	2
Intron 1b:								
ss38318594	IVS1b+28G→A	Gly56Ala[g]	3	NA	NA	NA	NA	NA
Intron 6:								
ss38318595	IVS6−44G→C	NA	1	2	1	0	2	0
Intron 7:								
ss38318596	IVS7+83C→T	NA	4	9	5	0	9	2
Intron 8:								
ss38318597	IVS8−33C→T	NA	2	4	2	0	4	0
Total			31	76	40	12	63	12

NOTE.—NA = not applicable.

[a] Changes in the cDNA are indicated relative to the GenBank *SLC6A4* reference sequence (accession number NM_001045); genomic variants are designated by +1 corresponding to the initiating ATG or position within an intron.

[b] Transmissions to all affected individuals, including the proband in whom the variant was first identified (T:NT = 76:12; $\chi^2 = 26.82$; 1 df; $P = 2.2 \times 10^{-7}$).

[c] Transmissions excluding the screened proband in whom the variant was initially identified (T:NT = 39:12; $\chi^2 = 8.13$; 1 df; $P = .0042$).

[d] Four 56Ala NTs derive from two heterozygous couples transmitting only one 56Ala allele to affected children.

[e] Redundant transmissions are not counted toward total transmissions.

[f] Family does not contribute to linkage; corresponding counts are not included in the totals.

[g] IVS1b+28G→A lies on the Gly56Ala allele; therefore, to avoid redundancy, transmission was not considered.

(NIMH) Center for Collaborative Genetic Studies on Mental Disorders repository. Lymphocytes were cultured in suspension in RPMI 1640 medium (supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a humidified incubator at 5% CO_2 prior to assay. Lymphocytes were pelleted at 1,500 rpm for 5 min and were washed with Krebs-Ringers-HEPES (KRH) assay buffer. A total of 1×10^6 cells in triplicate were prewarmed (37°C) in a shaking water bath (10 min) in 12 × 75 polypropylene tubes in KRH buffer that contained 100 μM pargyline and 100 μM ascorbic acid. After a 5-min incubation with [^{3}H]5-HT (20 nM) at 37°C, uptake assays were terminated by immersion on ice, and uptake in pelleted, 1% SDS-extracted cells was quantitated by scintillation spectrometry. Specific 5-HT uptake was determined by subtracting the amount of [^{3}H]5-HT accumulated in the presence of 10 μM paroxetine (SmithKline Beecham). [^{3}H]L-glutamate transport assays were conducted as described for [^{3}H]5-HT,

except with use of 100 nM substrate and definition of nonspecific uptake, with parallel accumulation acquired at 4°C.

Results

Linkage

On the basis of our initial linkage results (McCauley et al. 2004) and other reports of linkage to 17q (IMGSAC 2001*a*; Yonan et al. 2003), we substantially increased our sample of 73 Tufts/Vanderbilt and 85 AGRE families through acquisition from the NIMH repository; we reassessed evidence of linkage in the larger sample that contained 182 additional AGRE families. We found striking linkage in an overall data set of 341 multiplex families, with a peak recessive HLOD ($HLOD_{REC}$) score of 5.8 (fig. 1*A*) at two adjacent markers at ~53 cM (*D17S1800* [~1.4 Mb from *SLC6A4*] and *D17S1294* [~150 kb from *SLC6A4*]). Results from all parametric and NPL analyses are detailed in table 4. Influenced by two recent reports (Stone et al. 2004; Weiss et al. 2005), we incorporated a sex-specific approach in our linkage analysis of chromosome 17. We queried for male-specific effects by splitting the data set into (1) families containing only affected males (MO) and (2) the remaining female-containing (FC) families, similar to the approach of Stone and colleagues (2004). Linkage increased substantially in the MO sample ($HLOD_{REC} = 8.0$; $n = 202$), with negligible contribution from the 138 remaining FC families. The magnitude of linkage overall and in the MO families, extraordinary for a complex behavioral disorder, encouraged us to pursue more-detailed studies to identify autism-associated alleles within *SLC6A4*.

Association

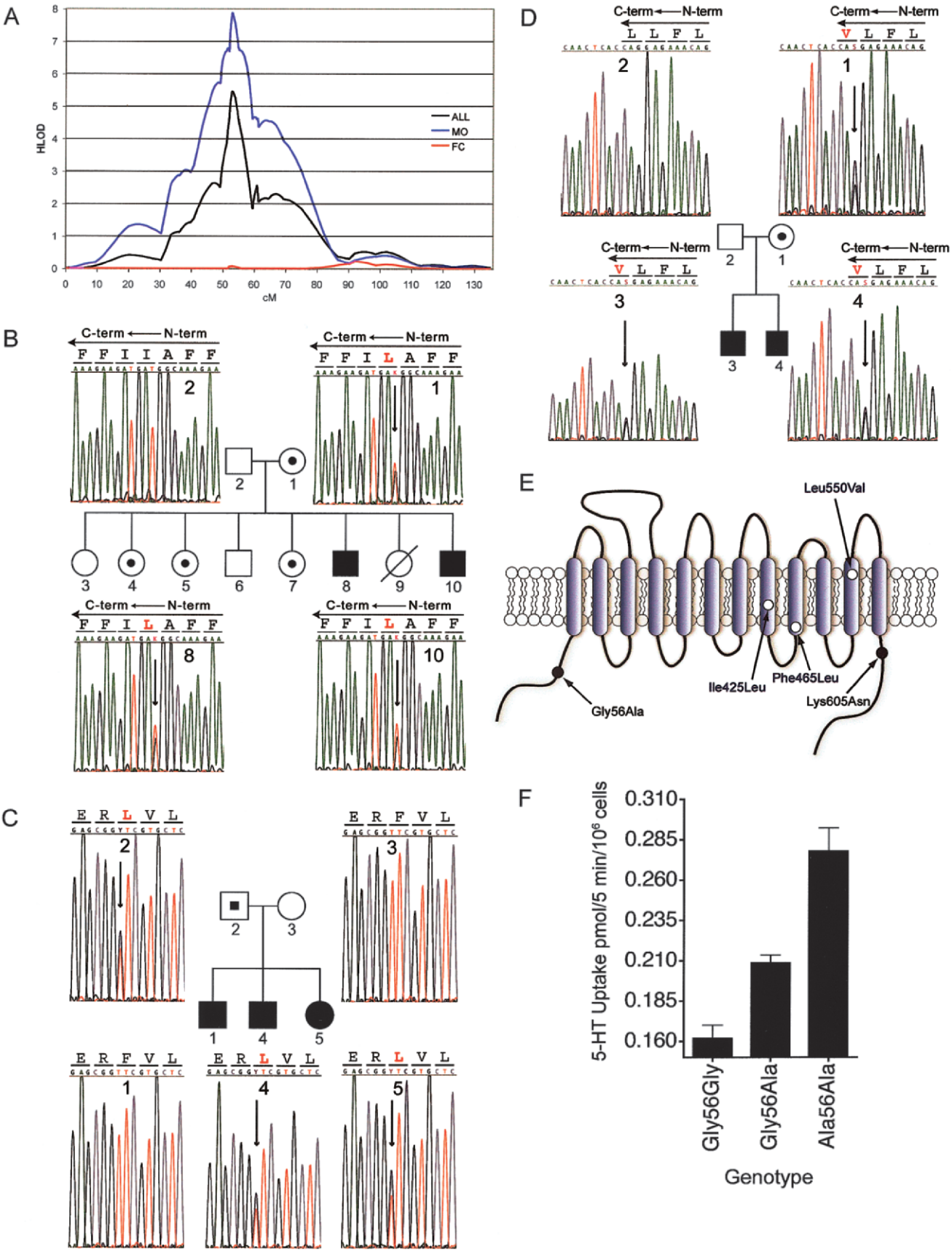
Initially, we tested for evidence of allelic association using the two markers (5-HTTLPR and the intron 5 SNP *rs140700*) that previously demonstrated modest association in a smaller sample (McCauley et al. 2004). In a sample of 384 combined multiplex and trio families, we detected evidence of allelic association with autism (i.e., transmissions [T] > nontransmissions [NT]) only at the intron 5 marker *rs140700* (minor allele T:NT = 93:124; $\chi^2 = 4.47$; $P = .03$). However, when MO and FC subsets were examined separately, modest association of the S allele of 5-HTTLPR was detected in the MO data set ($n = 235$ families; T:NT = 362:314; $\chi^2 = 4.90$; $P = .03$) as well as persistent undertransmission of the minor allele at *rs140700* (T:NT = 47:76; $\chi^2 = 6.84$; $P = .009$), whereas no association or trend toward association was seen in the 149 FC families (HTTLPR-S: T:NT = 223:231, $\chi^2 = 0.07$, $P = .79$; *rs140700*: T:NT = 46:48, $\chi^2 = 0.021$, $P = .88$). The association in MO families is consistent with previous results (McCauley et al. 2004) and the male bias in linkage (Stone et al. 2004), but it fails to explain the highly significant linkage in MO families shown above.

Allelic Heterogeneity at SLC6A4

Since *common* alleles across the *SLC6A4* locus do not explain the observed linkage, we considered the hypothesis that multiple, possibly rare, *SLC6A4* risk alleles exist and confer risk of autism. We tested our hypothesis using two parallel strategies. We first screened all families for known rare coding variants to determine if one or more is present at elevated frequency in our autism sample and, by inference, is potentially related to disease risk. Our second approach involved (1) selecting unrelated probands from the multiplex families that contribute most to linkage and, at minimum, have a positive LOD score at the linkage peak and (2) screening their *SLC6A4* exons and promoter sequences for novel variants. In our combined sample of 384 multiplex families and parent-child trios, we screened for known nonsynonymous variants using TaqMan allelic discrimination assays. We detected multiple individuals who carried one or two copies of the 56Ala-encoding allele and a single subject heterozygous for an allele encoding the nonsynonymous variant Lys605Asn.

Detailed analysis of families with the Gly56Ala substitution reveal its presence in both "linked" (positive LOD scores) and "unlinked" multiplex families and trios. Within the 120 families with the highest family-specific LOD scores, we found the 56Ala allele present on 11 (2.3%) of 480 independent chromosomes. Three homozygous 56Ala subjects were identified in these families. In contrast, Glatt et al. (2001) reported the 56Ala variant occurred on only 4 of 900 chromosomes in the only large *nonclinical* comparison sample described, with a minor-allele frequency (MAF) of 0.44% and no homozygous subjects. In our remaining families with autism, we found a lower frequency of the 56Ala allele, with 12 (1.1%) of 1,056 chromosomes harboring the variant; no homozygotes were identified for this group. An alternative comparison comes from our screening of a predominantly white population ascertained for Axis I Mood Disorders. Here, we identified 3 (1.1%) of 272 chromosomes with 56Ala alleles (J. R. Field, H.C.P., R.D.B., E. Sanders-Bush, and R. C. Shelton, unpublished findings).

A parallel strategy involved screening the promoter, exons, and flanking sequence of unrelated probands from, initially, 24 families who most contributed to linkage in this region, on the basis of ranking for family-specific LOD scores. TGCE followed by direct sequencing of PCR products comprised the initial effort, and one proband sample from each of the remaining 96 (of 120) families was subjected to direct sequencing of PCR prod-


A
HLOD
cM
ALL
MO
FC
B
C-term ← N-term
F F I I A F F
F F I L A F F
C
E R L V L
E R F V L
D
L L F L
V L F L
E
Leu550Val
Ile425Leu
Phe465Leu
Lys605Asn
Gly56Ala
F
5-HT Uptake pmol/5 min/10⁶ cells
0.310
0.285
0.260
0.235
0.210
0.185
0.160
Gly56Gly
Gly56Ala
Ala56Ala
Genotype

ucts. In addition to the independent identification of one 56Ala homozygote, two novel coding variants (Ile425Leu and Leu550Val) (fig. 1*B* and 1*D*) were detected in the 24 most-linked families (4/48 = 8.3% allele frequency). In the remaining 96 families, several 56Ala alleles and a Phe465Leu nonsynonymous substitution (fig. 1*C*) were detected (table 3). All three coding substitutions occur within transmembrane domains (fig. 1*E*) and are highly conserved (fig. 2). The least conserved of the novel variants corresponds to residue Ile425, which is present within all SERT proteins from human to *Drosophila*. The other two nonsynonymous variants occur at residues conserved from human to *Drosophila* in all monoamine transporters—including SERT, the norepinephrine transporter (NET), and the dopamine transporter (DAT)—for which sequence was available.

To consider the genetic relevance of the coding variants to autism risk, we asked if these variants segregated with disease in each family. Two of the three novel coding variants were transmitted to all affected individuals (5 males and 1 female) in the three families (fig. 1*B*–1*D*). One affected male did not inherit the paternally transmitted Phe465Leu variant allele. The 425Leu allele exhibited a particularly intriguing overall segregation pattern (fig. 1*B*). This variant was maternally transmitted to both affected sons. Three unaffected daughters also inherited the allele, but two other unaffected siblings, one male and one female, did not. When the clear sex bias in linkage is considered, segregation of the 425Leu allele in this family is consistent with but not proof of male-biased genetic risk or elevated penetrance associated with the allele. No unaffected siblings were present in the other two families. The 56Ala allele was detected in seven linked families, in which the T:NT was 15:5 (table 3). Of the five nontransmissions, four correspond to two distinct instances in which both parents were carriers (expected to occur in ~1 of every 2,000 couples, under the assumption of Hardy-Weinberg equilibrium [HWE]), and offspring received only one Ala56 allele. The other nontransmitted 56Ala allele was present in the mother of the family that paternally transmitted the Phe465Leu variant. Of the three homozygous individuals in the 120 linked/screened families, two were affected male offspring and one was a mother for whom medical history information was unavailable but who transmitted the allele to two affected male offspring. Analysis of the unlinked multiplex and simplex families with a 1.1% (12/1056) 56Ala frequency did not show bias in transmission (data not shown). There was a significant effect in a larger 643 multiplex/trio data set for autism in males carrying one or two 56Ala alleles compared with females (23:14 affected:unaffected males vs. 7:16 affected:unaffected females; $P = .016$).

Clinical Correlations

To explore phenotypic correlates with the coding variants, we compared scores for trait subsets of autism that were based on ADI-Revised (ADI-R)–derived variable clusters (table 5) identified elsewhere from a principal-components analysis of ADI-R items (Tadevosyan-Leyfer et al. 2003). These clusters reflect (1) language, (2) social intent, (3) developmental milestones, (4) savant skills, (5) rigid-compulsive, and (6) sensory-aversion aspects of the autism phenotype. There was a significant increase in rigid-compulsive behaviors associated with the novel variants ($P = .0003$) (table 5), and the effect was most pronounced for the Ile425Leu and Leu550Val families. The Ile425Leu substitution tracked with more-severe language deficits ($P = .0031$), although the brothers harboring the allele were generally more affected across most factor domains, with the exception of sensory aversions. The Leu550Val and Phe465Leu variants were associated with lesser impairment in language and social-intent domains, significantly so for the Phe465Leu variant ($P < .0001$ and $P = .0021$, respectively). The Gly56Ala variant (heterozygous or homozygous) in the linked families similarly demonstrated a significant as-

Figure 1 Male-biased linkage of autism and novel coding variants at the 17q11.2 *SLC6A4* locus. *A,* Male-biased linkage of autism to 17q11.2. Multipoint linkage analysis on chromosome 17 is shown for the overall 341-family data set (*black line*), 202 MO families (*blue line*), or the remaining 138 FC families (*red line*). HLOD scores were calculated under a recessive model and were plotted as a function of marker position in centimorgans (cM) along chromosome 17. *B–D,* Sequence detection of novel nonsynonymous *SLC6A4* variants in families with autism. Sequence-based detection is shown for each of the three novel coding variants, with corresponding pedigrees. *B,* Ile425Leu. *C,* Phe465Leu. *D,* Leu550Val. Blackened circles or squares reflect individuals with an autism diagnosis, unblackened circles or squares reflect individuals without autism, and allele carriers without autism are indicated by small blackened circles or squares within the larger pedigree symbol. Electropherogram data is shown in either sense (*B*) or antisense (*A* and *C*) orientations, with corresponding coding sequence. Antisense sequences (*A* and *C*) indicate the reversed orientation of amino acid codons, represented by lines across each three-base sequence. Variant amino acids are shown in red, and corresponding heterozygous sequence changes are indicated by an arrow. Individual numbers in the respective pedigrees correspond to numbers within each of the sequence frames. *E,* Schematic representation of the 5-HT transporter. Amino acid substitutions are indicated by location within transmembrane or cytoplasmic domains. *F,* Dosage-dependent elevated 5-HT transporter activity of Ala56-encoded hSERT in native lymphoblastoid cells. Lymphocytes genotype-matched at 5-HTTLPR (L/L) and the intron 2 VNTR (10/10) and bearing Gly/Gly–, Gly/Ala–, or Ala/Ala–encoding genotypes at residue 56 were assayed for [^{3}H]5-HT transport activity, as described (see the "Subjects and Methods" section). Three independent experiments were performed in triplicate for each line, and the combined basal uptake data were plotted.

sociation with more severe rigid-compulsive behaviors ($P = .0085$). When all linked families with individuals carrying a coding variant were considered together, the significance in elevated severity of rigid-compulsive behaviors increased ($P = .0002$). No consistent pattern was observed across all Gly56Ala families, regardless of sex, for other ADI clusters; however, there appeared to be a trend toward two subgroups. In one group, patients had greater severity for rigid-compulsive and sensory-aversion behaviors, with fewer impairments in language and social domains. The second group did not show the sensory-aversion finding but was more impaired in language (table 5).

Functional Properties of Gly56Ala SERT

The presence of multiple homozygous 56Ala subjects in our multiplex autism sample allowed evaluation of the functional impact of a 56Ala substitution on SERT activity and regulation by use of genotyped, EBV-transformed lymphocytes, since *SLC6A4* is natively expressed in those cells (Khan et al. 1996; Lesch et al. 1996; Faraj et al. 1997). Basal 5-HT transport activity was elevated in Ala56-expressing cells (T test, $P < .05$) compared with Gly56 homozygous, Gly56Ala heterozygous, or combined Gly56 genotypes; significance remains after normalization to L-glutamate transport activity and does not segregate with the *SLC6A4* genotype (fig. 3). To control for potential confounding effects of variation in *SLC6A4* gene expression and downstream basal 5-HT uptake associated with 5-HTTLPR and intron 2 VNTR genotypes (Lesch et al. 1996; Ogilvie et al. 1996; MacKenzie and Quinn 1999), we repeated studies with two cell lines of each Gly56Ala genotype that carried identical 5-HTTLPR (L/L) and intron 2 VNTR (10/10) genotypes. Figure 1*F* demonstrates a 56Ala dosage-dependent effect on basal 5-HT transport activity, with ~75% increase evident for the 56Ala homozygous lines as compared with lines homozygous for 56Gly. In these cells, we also demonstrated that the 56Ala allele is refractory to regulation by acute application of activators of protein kinase G (PKG) or p38 mitogen-activated protein kinase (MAPK) (Miller and Hoffman 1994; Qian et al. 1997; Ramamoorthy and Blakely 1999; Zhu et al. 2004, 2005; Samuvel et al. 2005) (fig. 4*A* and 4*B*), which suggests that intrinsic features of function unlikely to be attributable to a linked, noncoding variant are evident. We recently described a similar loss of regulation despite normal surface density for 56Ala cDNA transfected into HeLa cells (Prasad et al., in press).

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 2 ClustalW alignment of hSERT amino acid sequence versus other species of SERT and other biogenic amine transporters. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Association of Heterogeneous Alleles with Autism

To more completely evaluate the *SLC6A4* locus for novel alleles, we expanded our screen to cover proximal promoter sequences and noncoding 5′ and 3′ exons. We subsequently analyzed 31 families for allelic segregation patterns for rare, noncoding variants discovered across the locus. Variants were identified in the promoter, exon 1b, exon 14, and intronic sequences in the linked families (table 3). Several variants are known polymorphisms with existing database identifiers, although many are novel. Other polymorphisms with modest-to-high MAF were tested elsewhere for association and are not included in table 3. Allelic segregation patterns reveal a stark pattern of transmission disequilibrium (TD) when all variants are combined for purposes of considering segregation. Redundant allelic transmissions represented by two or more SNPs were not counted more than once. TD was evident from a T:NT count of 76:12, which represents a highly significant deviation from the null hypothesis of no association ($\chi^2 = 26.82$; 1 df; $P = 2.2 \times 10^{-7}$). There is an a priori expectation of transmission (or de novo sequence change) to index cases in whom variants were originally detected. To reduce this bias, transmissions to these individuals were excluded, yet segregation remained significant (T:NT = 40:12; $\chi^2 = 8.13$; 1 df; $P = .0042$). Therefore, these multiple coding and noncoding alleles persist in demonstrating a collective link-

Table 4

Autism Linkage Data for Chromosome 17

Family Sample	Marker	Position[a] (cM)	HLOD		LOD*	LOD* P[b]	NPL	NPL P[b]
			Dominant	Recessive				
Overall ($n = 340$)	*D17S1800*	53	4.98	5.44	5.82	1.59×10^{-7}	4.88	6.63×10^{-8}
MO ($n = 189$)	*D17S1800*	53	5.35	7.86	6.65	4.85×10^{-8}	5.18	1.64×10^{-8}
FC ($n = 138$)	*D17S1800*	53	.4	.06	.46	.085	2.21	.073

[a] Position on the deCODE chromosome 17 genetic map.
[b] P values are nominal and are not corrected.

Table 5

ADI Cluster Scores for Families with *SLC6A4* Coding Variant

		Mean for Sample				All Novel Variants		No. of Subjects with	Mean for Carriers with						All G56A Carriers		Male Gly56Ala Carriers			All Coding	
ADI Cluster	No. of Subjects	Total	Ile425Leu	Leu550Val	Phe465Leu	Mean	*P*	G56A	G56A-1	G56A-2	G56A-3	G56A-4	G56A-5	G56A-6	Mean	*P*	No. of Subjects	Mean	*P*	Mean	*P*
Language	770	***.457 ± .268***	***.838***[a]	.314	***.231***[b]	.461 ± .329	NS	13	.647	.378	.552	.207	.378	.436	.462 ± .156	NS	9	.541 ± .297	NS	.442 ± .205	NS
Social Intent	770	***.439 ± .228***	***.605***[c]	.325	***.200***[d]	.377 ± .207	NS	13	.564	.164	.314	.454	.165	.344	***.334 ± .158***	.075	9	.444 ± .203	NS	.370 ± .169	.095
Milestones	758	***.738 ± .068***	.809	.740	.763	.771 ± .035	.052	13	.723	.734	.750	.780	.757	.759	.742 ± .026	NS	9	.754 ± .056	NS	***.757 ± .026***	***.0069***
Savant Skills	730	***.124 ± .150***	.000	.083	.028	***.037 ± .042***	***.0031***	13	.088	.671	.111	.033	.671	.109	.215 ± .238	NS	9	.150 ± .157	NS	.199 ± .270	NS
Rigid Compulsive	747	***.288 ± .209***	.402	.428	.351	***.394 ± .039***	***.0003***	13	.317	.412	.210	.497	.413	.606	***.409 ± .138***	***.0085***	9	***.343 ± .138***	NS	***.404 ± .11***	***.0002***
Sensory Aversion	708	***.331 ± .269***	.060	.245	.340	.215 ± .142	.08	11	.305	.667	.319	.667	.667	NA	***.525 ± .195***	***.0005***	7	.353 ± .272	NS	.409 ± .231	NS

NOTE.—Values shown in bold italics are statistically significant ($P < .05$) in comparison with overall means. NS = not significant.

[a] Ile425Leu-Language mean = .838 ± .34; $P = .0031$.

[b] Social Intent mean = .605 ± .026; $P = .011$.

[c] Phe465Leu-Language mean = .231 ± .0035; $P < .0001$.

[d] Social Intent mean = .200 ± .06; $P = .0021$.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 3 Altered basal 5-HT transport activity and regulation associated with the Gly56Ala variant. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics.*

age to and allelic segregation with autism, despite failure to identify association of *common* alleles (apart from HTTLPR and *rs140700*) at *SLC6A4* in these families.

Discussion

Two recent reports are notable for leveraging the sex bias of disease affection in autism to define an analytical framework that identified significant male-biased genetic effects at 17q. The AGRE Consortium reported a genomewide analysis with use of sex (as presented here) to stratify a 257-family data set (Stone et al. 2004), and they have recently replicated evidence for male-biased linkage to 17q (Cantor et al. 2005). They compared overall linkage with that of MO families and of FO families. They found a significant effect at 17q11.2 near the *SLC6A4* locus (~53 cM), with MO families showing an empirically significant increase of linkage from a maximum LOD score (MLS) of 3.2 in the overall data set to an MLS of 4.3 in the 148-family MO data set. Our analyses, which include AGRE and Tufts/Vanderbilt families, confirm these previous observations by the AGRE Consortium yet show more-pronounced overall and male-biased genetic effects at this locus. In the second report, Weiss et al. (2005) showed significant association of marker alleles at *SLC6A4* and the 17q31 integrin $\beta 3$ (*ITGB3* [MIM 173470]) locus, with male-specific genetic influences on 5-HT levels. *ITGB3* was detected earlier by Weiss et al. (2005) as a QTL for circulating serotonin levels. Our own recently published genomic linkage screen showed two adjacent linkage peaks on proximal 17q (McCauley et al. 2005). Conditional linkage analyses revealed locus- or peak-specific effects, which supports the premise that both *SLC6A4* at 53 cM and another more telomeric locus (possibly *ITGB3*) contribute to genetic effects in this region. We speculate on the basis of published linkage patterns—including the AGRE replication study that showed linkage (MLS = 3.6) to a more distal site in 17q21 (Cantor et al. 2005)—that overall linkage in this region represents two loci, with the likely stronger effect at *SLC6A4*.

We were struck by the extraordinary evidence of linkage, given that common alleles and haplotypes fully representing LD across the locus failed to explain the linkage, as would be predicted under the "common disease-common allele" hypothesis. Given the above observations, we considered the alternative hypothesis that allelic heterogeneity explained risk at *SLC6A4*. We have discovered three novel highly conserved coding variants in families that strongly contribute to linkage in this region. Two of the three novel coding variants (Ile425Leu and Leu550Val), a 56Ala homozygous proband, and a novel intron 6 SNP (IVS6-44G→C) were identified among 24 probands (8.3% allele frequency) from families with the highest family-specific LOD scores. These two novel alleles segregate to all affected individuals in their respective families and thus are associated with disease. These findings spawned further discovery efforts that led to the identification of 13 novel SNPs, including the Phe465Leu nonsynonymous variant. The family with this latter variant is of interest because the mother carried but did not transmit the 56Ala allele. She did trans-

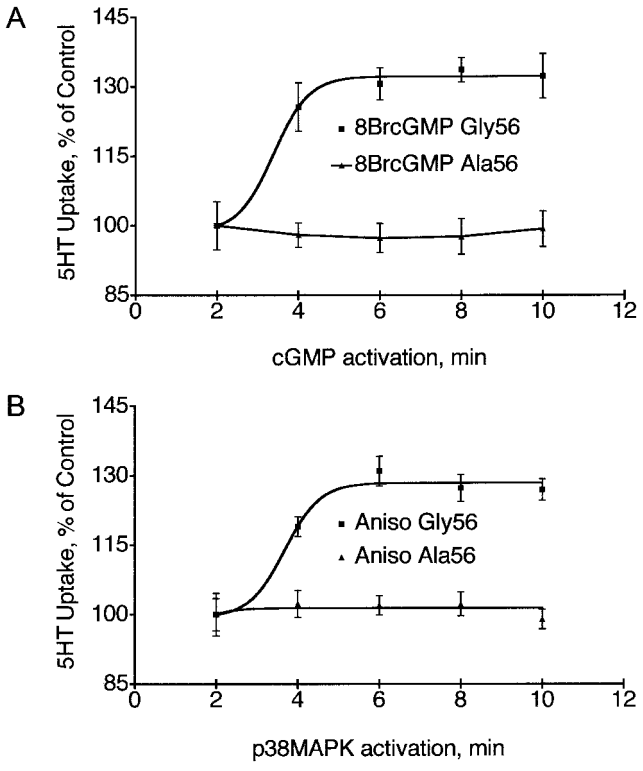


Figure 4 SERT is refractory to regulation through PKG and p38 MAP kinase signaling pathways. *A*, SERT Ala56 lacks sensitivity to 8BrcGMP. Homozygous Ala56 cells (10^6/tube) were preincubated for various lengths of time at 37°C, with 10 μM 8BrcGMP prior to [^{3}H]5-HT transport assays. *B*, SERT Ala56 lacks sensitivity to the p38 MAPK activator anisomycin; 8BrcGMP effects on Gly56 SERT are completely blocked by coincubation with the PKG inhibitor H8 (10 μM), whereas the p38 MAPK inhibitor SB203580 (1 μM) prevented anisomycin stimulation (data not shown). Data plotted represent mean data ± SD (n = 3) for a single cell line of each genotype assayed in parallel. Findings were replicated with identical results in an additional line for each genotype.

mit a rare genomic variant (hCV11414114) (table 3) to all affected children. The study by Glatt and colleagues (2001) did not detect the three novel coding alleles in the 450 individuals (900 chromosomes) sequenced; this indicates that these alleles are rare and have a frequency of <1/900, or <0.11%.

Cross-species conservation of novel coding-variant residues and their identification in linked families suggests an increased likelihood of altered functionality for the variant transporter, although future study will be required to fully elaborate this premise. All three novel substitutions occur within transmembrane domains (fig. 1*E*), and the 425Leu allele affects the identical residue and nucleotide—as does the Ile425Val mutant in the pedigrees described by Ozaki and colleagues (2003)—that segregates Asperger syndrome (MIM 608638), obsessive-compulsive disorder (OCD [MIM 164230]), and other psychiatric phenotypes. The Ile425Leu variant does, therefore, have an a priori increased likelihood of functional effect that is based on prior precedent from disease association and subsequent functional characterization of the Ile425Val substitution showing ~2-fold elevated basal activity in transfection studies (Kilic et al. 2003; Prasad et al., in press).

The Gly56Ala substitution shows a very suggestive increase of MAF to 2.3% in the 120 linked families, compared with the 1.1% seen in the remaining families. A 2.3% allele frequency represents a noticeable increase over the single *nonclinical* reference study (Glatt et al. 2001), which shows 4 of 900 (0.44%) chromosomes (and no homozygotes) carrying a 56Ala allele. HWE would dictate a frequency of homozygous individuals to be ~1/2,000 in unrelated individuals (or ~1/5,000 on the basis of a 0.44% frequency in the Glatt et al. [2001] comparison sample). Therefore, our finding of three homozygotes and two additional (unrelated) instances of dual heterozygous couples is highly unlikely by chance ($P \approx 0$). The findings of (1) an apparent fivefold increase in 56Ala allele frequency in the linked families, with a trend toward overtransmission, (2) a substantial deviation from HWE, and (3) a male-biased trend toward autism affection in the presence of this allele compared with females indicates a role for the 56Ala allele as a genetic risk factor in autism. The infrequency of the allele, however, makes this more difficult to quantitate. Larger autism population studies are indicated.

Equally important are functional data that demonstrate that the 56Ala SERT protein displays an elevated basal activity and insensitivity to regulation through PKG and p38 MAPK signaling pathways in a native cell system. Importantly, down-regulation in response to protein kinase C–activating phorbol esters was equivalent between Gly56 and Ala56 lines (data not shown). We do not believe that PKG/p38 MAPK regulatory insensitivity derives directly from altered basal activities, since we have demonstrated transport regulation through these pathways with other variants that differed up to 10-fold in activity (Prasad et al., in press), although over-expression in transfected cells can blunt regulation (C.-B. Zhu and R.D.B., unpublished findings). Elevated basal 5-HT uptake is also not due to enhanced transcription, since RealTime PCR analysis revealed equal SERT mRNA levels in the Gly56 and Ala56 lines (data not shown). Elevated basal activity of the Ala56-encoded transporter is intriguing in light of a similar effect with 425Val-encoded SERT in patients with Asperger syndrome and OCD. Although altered regulation was not seen with the Ile425Val mutation, the elevated basal transporter activity may suggest a common functional mechanism important in autism, OCD, and other phenotypes. It is possible that prolonged expression of a variant lacking regulation induces other compensatory changes that influence basal transporter activity. Regardless, these findings suggest that Gly56Ala carriers may possess an *inflexibility to regulatory stimuli* that could thereby compromise appropriate demand-dependent modulation of SERT surface expression and/or catalytic activity.

Phenotypically, the three novel variants and Gly56Ala are significantly associated with increased rigid-compulsive behaviors. These include (1) stereotyped utterances, (2) unusual preoccupations, (3) compulsions/rituals, (4) resistance to trivial changes in the environment, and (5) unusual attachment to objects (Tadevosyan-Leyfer et al. 2003). This is important for several reasons. Our previous study of linkage in a smaller sample demonstrated a significant increase in linkage at ~53 cM when the same rigid-compulsive trait was used to stratify the data set (McCauley et al. 2004). It is consistent with the finding of Asperger syndrome, OCD, and other traits in families segregating the functionally abnormal (Prasad et al., in press) Ile425Val substitution (Ozaki et al. 2003). Obsessive-compulsive–type traits and clinical OCD are seen more frequently in families with autism than in the general population (Bolton et al. 1998). Repetitive behaviors and associated anxiety in autism and OCD are often effectively treated with SSRIs, targeting the SERT protein (Hollander et al. 2005). Whereas other phenotypic findings associated with specific variants were variable across families with all four variants, increased severity for the rigid-compulsive domain was a consistent finding for these variants. Given the magnitude of the observed genetic effect, the possibility exists that our sample may be enriched by chance or selection methods for phenotypic traits (or regional alleles) that bias in favor of an effect at this locus.

The functionality of promoter and other noncoding variants is unknown; however, the segregation of these multiple variants in aggregate, in addition to the coding variants, provides additional genetic evidence of an allelic heterogeneity framework for disease risk involv-

ing *SLC6A4*. Several previously documented variants (*rs2020932,* hCV11414117, and hCV11414114) were each found in multiple families. These heterogeneous variants arose largely on independent haplotypic backgrounds, which indicates that they are rare independent events and are not the result of an effect of some common genetic background. The clustering of collectively associated rare genomic variants in the promoter and near the 5′ end of the gene raises the possibility of transcriptional effects at *SLC6A4*. Intronic or noncoding transcribed variants may, if they are risk factors with biological relevance, affect transcription, transcript stability, or RNA splicing (Pagani and Baralle 2004). An expression-based mechanism for potentially disease-related 5′ variants is consistent with association of the HTTLPR marker, since the insertion/deletion variant exhibits differential transcription. Even with a relatively conservative analysis of transmission by eliminating the subject from each family in whom the variant was discovered, association data in the presence of linkage must be interpreted cautiously. The analysis of transmissions in families with an a priori expectation of variable allele-sharing would inflate any measure of allelic association, and the TDT analysis of *multiplex* families in this unusual context would yield a measure of linkage (Spielman and Ewens 1996). Nevertheless, the context in which these observations are made supports the significance of association in the aggregate of these multiple variants. The modest association at 5-HTTLPR and the intron 5 SNP *rs140700* in the current MO sample is not only consistent with but is also supportive of this proposed risk framework. Other common alleles are not associated with autism, whereas the heterogeneous rare alleles described in this report are. Thus, the most parsimonious model involves multiple different risk alleles (including 5-HTTLPR) that act in different families to collectively account for the observed linkage.

The import of our data is summarized by the constellation of findings, including (1) three novel highly conserved coding variants, one of which affects a residue (Ile425) with known phenotypic and functional relevance; (2) the dysfunctional properties of SERT encoded with the Gly56Ala substitution; (3) the stark deviation from HWE and increased frequency in linked families; (4) a phenotypic correlation between coding variants and increased rigid-compulsive behaviors; and (5) the aggregate association shown by heterogeneous promoter 5′ and intragenic noncoding variants. These data collectively support the premise that *SLC6A4* represents a susceptibility locus for autism-spectrum disorders. Our findings must be examined in larger independent autism family populations with similar phenotypic representation to determine their ultimate significance. Given the linkage reported at *SLC6A4* by IMGSAC (as well as AGRE) and incomplete screening in the current sample (only one affected individual per family was screened for exon and promoter variants), we suspect that additional coding and noncoding variants will be discovered at this locus. As they stand now, our findings provide compelling evidence that the *SLC6A4* locus is a bona fide autism-susceptibility gene, with variants predisposing to rigid-compulsive traits.

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Pevsner, Ph.D., Carol Sprouse, Ed.D., Gerard Schellenberg, Ph.D., and Rudolph Tanzi, Ph.D.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC6A4* [accession number NM_001045])
NIMH Center for Collaborative Genetic Studies on Mental Disorders, http://www.nimhgenetics.org/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for autism, *SLC6A4, ITGB3,* Asperger syndrome, and OCD)
Single Nucleotide Polymorphism, http://www.ncbi.nlm.nih.gov/SNP/ (for dbSNP numbers *ss38318598, ss38318599, ss38318600, ss38318601, ss38318589, ss38318590, ss38318591, ss38318592, ss38318593, ss38318594, ss38318595, ss38318596,* and *ss38318597*)

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