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A network of dopaminergic gene variations implicated as risk factors for schizophrenia

Michael E. Talkowski[1,3], George Kirov[4], Mikhil Bamne[1], Lyudmila Georgieva[4], Gonzalo Torres[2], Hader Mansour[1], Kodavali V. Chowdari[1], Vihra Milanova[5], Joel Wood[1], Lora McClain[1], Konasale Prasad[1], Brian Shirts[3], Jianping Zhang[6], Michael C. O'Donovan[4], Michael J. Owen[4], Bernie Devlin[1,3] and Vishwajit L. Nimgaonkar[1,3,*]

[1]Department of Psychiatry and, [2]Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, [3]Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA, USA, [4]Department of Psychological Medicine, Cardiff University, Heath Park, Cardiff, UK, [5]Department of Psychiatry, Medical University, Sofia, Bulgaria and [6]Division of Clinical Pharmacology, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Medical School, Mayo Clinic, Rochester, MN, USA

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We evaluated the hypothesis that dopaminergic polymorphisms are risk factors for schizophrenia (SZ). In stage I, we screened 18 dopamine-related genes in two independent US Caucasian samples: 150 trios and 328 cases/501 controls. The most promising associations were detected with *SLC6A3* (alias DAT), *DRD3*, *COMT* and *SLC18A2* (alias VMAT2). In stage II, we comprehensively evaluated these four genes by genotyping 68 SNPs in all 478 cases and 501 controls from stage I. Fifteen (23.1%) significant associations were found ($p \leq 0.05$). We sought epistasis between pairs of SNPs providing evidence of a main effect and observed 17 significant interactions (169 tests); 41.2% of significant interactions involved rs3756450 (5′ near promoter) or rs464049 (intron 4) at *SLC6A3*. In stage III, we confirmed our findings by genotyping 65 SNPs among 659 Bulgarian trios. Both *SLC6A3* variants implicated in the US interactions were overtransmitted in this cohort (rs3756450, $p = 0.035$; rs464049, $p = 0.011$). Joint analyses from stages II and III identified associations at all four genes ($p_{joint} < 0.05$). We tested 29 putative interactions from stage II and detected replication between seven locus pairs ($p \leq 0.05$). Simulations suggested our stage II and stage III interaction results were unlikely to have occurred by chance ($p = 0.008$ and 0.001, respectively). In stage IV we evaluasted rs464049 and rs3756450 for functional effects and found significant allele-specific differences at rs3756450 using electrophoretic mobility shift assays and dual-luciferase promoter assays. Our data suggest that a network of dopaminergic polymorphisms increase risk for SZ.

INTRODUCTION

The distribution of schizophrenia (SZ) in families and populations is consistent with a substantial genetic basis for the disorder. No obvious genetic model can explain the data, but models including multiple interacting loci conferring risk provide a good fit (1,2). The disorder is common, with an estimated lifetime morbid risk of 1%, and concordance estimates for monozygotic twins (48%) are significantly higher than that for dizygotic twins (17%) (3). There has been long-standing research into the hypothesis that dopamine dysfunction contributes to SZ pathogenesis (4,5). The hypothesis originated from observed correlations between the clinical potency of anti-psychotic drugs and their affinity for dopamine D2 receptors (*DRD2*) (6–8). Patients with SZ display increased sensitivity to the psychotogenic effects of agents that increase synaptic dopamine release (5,9–12). In addition, acute amphetamine challenge to SZ patients leads to increased

* To whom correspondence should be addressed at: Department of Psychiatry, University of Pittsburgh School of Medicine, WPIC, Office 444, 3811 O'Hara Street, Pittsburgh, PA 15213, USA. Tel: +1 4122466353; Fax: +1 4122466350; Email: nimga@pitt.edu

dopaminergic transmission *in vivo*, as measured by radioligand binding to dopamine D2 receptors during positron emission tomography scans (13–15). Therefore, dopamine genes have traditionally been prime candidates for genetic studies in SZ.

Despite the substantial biological evidence implicating dopaminergic dysfunction in SZ pathogenesis, it is not precisely known whether genetic polymorphisms in dopaminergic genes are associated with dopamine abnormalities. If such a functional link exists, the nature of these variations, the number of genes affected, interactions among them and their functional importance are poorly understood. Associations between SZ and many dopaminergic gene variations have been reported, but most studies evaluated one or at best a handful of polymorphisms, usually on the basis of preliminary evidence of a functional impact (e.g. exonic SNPs or functional repeats). Most previous studies were better suited to identify risk factors of substantial effect size than multiple interacting loci, for which the marginal effect of an individual locus could be small. Therefore, it appears that many genes in the dopamine pathway have not been investigated adequately for their impact on SZ risk. Our recent review of the literature estimated that ~5% of representative common SNPs currently available in public databases have been considered in association studies of dopaminergic genes with at least 50% power to detect modest effect sizes expected [odds ratios (OR) from 1.2 to 1.5] (16). For example, a large number of studies investigated a single coding variant (rs6280) at the dopamine D3 receptor gene (*DRD3*) with largely inconsistent results (17–19). Until recently, studies did not consider other variations within the gene. Two independent studies of 13 SNPs and 17 SNPs now suggest that associations with other SNPs/haplotypes might account for past inconsistencies at rs6280 (20,21). Similar associations could be present with common variants yet to be investigated at other dopaminergic targets, but alternative strategies may be necessary to jointly evaluate these genes.

Multi-stage studies can be useful in analyses of a functionally related network of genes by initially screening a large group of susceptibility targets and subsequently evaluating only the most promising candidates in additional samples, thus maximizing power with the resources available (22–24). Skol *et al*. (25) recently showed that an increase in power for multi-stage whole-genome studies can be attained by evaluating the joint distribution of test statistics from both samples versus independent consideration of each sample. We reasoned a similar approach could be applied to gene-based association studies that are restricted to a smaller number of loci, since samples from individual studies are almost always underpowered to consistently detect associations and interactions of modest effect. In the present study, we revisited the genetic basis for the so-called 'dopamine hypothesis' of SZ by investigating 18 dopaminergic genes in three independent samples. We hypothesized that key susceptibility variants within the dopaminergic network could be identified if results from multiple samples were evaluated jointly. Our multi-stage strategy progressively pruned the list of promising susceptibility candidate genes and culminated in functional analyses of associated SNPs.

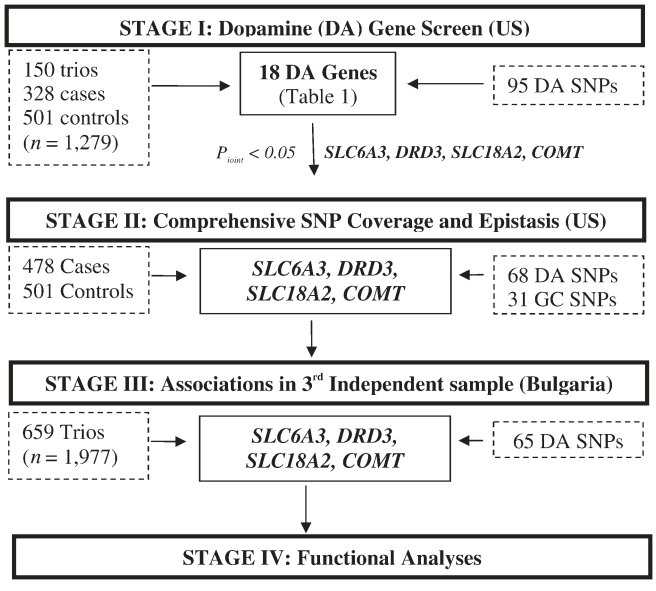

Figure 1. Study design. Overview of multi-stage study design utilized, including all samples and SNPs analyzed in each stage. In stage I, the 328 cases are independent of the 150 probands from the family-based samples. DA, dopamine; GC, genomic control.

RESULTS

Design overview

An overview of the study design is provided in Figure 1. Briefly, in stage I, we screened 18 dopamine-related genes, using two independent samples from the USA: a family-based sample and a case–control sample. To improve the power of our screen, we evaluated the joint distribution of test statistics from both samples. In stage II, in-depth analyses of the most promising stage I genes were conducted using tag SNPs and all available case–control samples from stage I. Pair-wise epistatic interactions were then modeled for a limited number of SNPs in which evidence for main effects were detected. In stage III, we analyzed a third independent sample from Bulgaria. In sum, 3256 participants were genotyped. Finally, functional effects of key SNPs were examined in stage IV.

Candidate genes

Because the list of genes impacting dopaminergic function is potentially long, subjective and continually expanding, we restricted our evaluation to dopaminergic genes analyzed in genetic association studies as of 2003. The selected genes included those required for dopamine synthesis (*TH, DDC*), transport (*SLC6A3, SLC6A2, SLC18A1, SLC18A2*), metabolism (*MAOA, MAOB, COMT*), conversion of dopamine to norepinephrine (*DBH*) and all dopamine receptors (*DRD1, DRD2, DRD3, DRD4, DRD5*) (Table 1). We also chose three genes important for dopamine regulation, namely *PPP1R1C* (alias DARPP-32), *DRD1IP* (alias CALCYON, a dopamine D1 receptor-interacting protein) and *NR4A2* (alias NURR1, an orphan nuclear receptor and putative transcription factor for the dopamine transporter) (Table 1). One candidate, *DRD3*, was analyzed in our US sample earlier as part of a

Table 1. Dopaminergic genes and SNPs analyzed

Gene	Location	Gene name (alias name)	Size (kb)	SNPs genotyped		
				Stage I	Stage II	Stage III
COMT	**22q11.2**	**Catechol-*O*-methyltransferase**	**27.2**	**7**	**18**	**17**
DBH	9q34	Dopamine beta hydroxylase	23.0	9		
DDC	7p11	Dopamine decarboxylase	102.6	5		
DRD1	5q35.1	Dopamine D1 receptor	3.1	3		
DRD1IP	10q26.3	D1 receptor-interacting protein (CALCYON)	11.5	5		
DRD2	11q23	Dopamine D2 receptor	65.6	5		
DRD3	**3q13.3**	**Dopamine D3 receptor**	**50.2**	**13**[a]	**18**	**18**
DRD4	11p15.5	Dopamine D4 receptor	3.4	3		
DRD5	4p16.1	Dopamine D5 receptor	2.1	3		
MAOA	Xp11.3	Monoamine oxidase A	90.6	10		
MAOB	Xp11.3	Monoamine oxidase B	115.8	6		
NR4A2	2q24.1	Orphin nuclear receptor subunit 4 (NURR1)	8.3	5		
PPP1R1B	17q21.2	Protein phosphatase 1, regulatory (inhibitory) subunit 1B (DARPP-32)	9.7	4		
SLC18A1	8p21.3	Vessicular monoamine transporter, member 1 (VMAT1)	38.4	10		
SLC18A2	**10q25**	**Vessicular monoamine transporter, member 2 (VMAT2)**	**35.9**	**3**	**14**	**13**
SLC6A2	16q12.2	Monoamine transporter, noradrenaline (NET)	46.0	8		
SLC6A3	**5p15.3**	**Dopamine transporter (DAT, DAT1)**	**52.6**	**6**	**18**	**17**
TH	11p15.5	Tyrosine hydroxylase	7.9	3		

Dopamine genes and SNPs analyzed are given in alphabetical order. The boldfaced genes were further analyzed in stages II and III.
[a]These SNPs were previously analyzed and results from those published analyses suggested significant associations in these samples (20).

collaborative study (20). On the basis of the significant associations detected in that study, *DRD3* was retained for stage II of this study, which included 501 independent controls.

Stage I: SNP screen among two independent US samples

We conducted 95 tests of association in the US family-based sample (150 trios, SNPs selected from Celera, 2003 based on physical distance). The most significant association was detected at *SLC6A3* (DAT) (rs403636, $p = 0.0004$, OR = 2.36). Transmission distortion was noted at two other *SLC6A3* SNPs (rs27072, $p = 0.001$; rs12516948, $p = 0.07$). All trends for association ($p < 0.10$; $n = 9$ SNPs) were genotyped in a replicate US case–control sample (328 cases, 501 controls). In this independent sample, significant associations were detected with four SNPs, including replication of rs403636 ($p = 0.04$). The joint distribution of test statistics from both samples identified *SLC6A3, DRD3, COMT* and *SLC18A2* as the four most promising candidates ($p_{\text{joint}} < 0.05$) (see Table 2 for selected significant results; complete list in Supplementary Material, Table S1). These four genes were retained for follow-up analyses.

Stage II: comprehensive gene coverage and epistasis among US samples

We assayed 68 SNPs among all available cases and controls from stage I (478 cases, 501 controls) at *SLC6A3, DRD3, COMT* and *SLC18A2*. SNPs were obtained from HapMap (26) and in-house sequencing for SNP detection. These analyses were not intended to replicate the stage I findings, as the samples overlapped. Instead, they enabled us to conduct in-depth analysis of representative common variants (minor allele >5%) from these four genes, over and above what was possible in our initial screen.

Overall, the distribution of test statistics from these SNPs was skewed towards small *p*-values (median trends test 1.07; expected median 0.456). Significant associations ($p < 0.05$) were found for 15 SNPs (Supplementary Material, Table S2). At *SLC6A3*, six of 17 SNPs tested were nominally significant ($p < 0.05$). Linkage disequilibrium (LD) analyses revealed that these associated SNPs were not part of a single cluster (Fig. 2). Associations were also detected with six *DRD3* SNPs, three *SLC18A2* SNPs and one *COMT* SNP.

Gender-specific analyses were conducted at three *COMT* SNPs on the basis of a previously reported association by Shifman *et al*. (27). Consistent with those findings, logistic regression revealed a significant interaction between gender and rs737865 genotype ($\chi^2 = 14.14$, 2 d.f., $p = 0.0007$). The significant effect appeared to be attributable to females, and a trends test comparing female patients with female controls for this SNP revealed significant differences in genotype distributions between groups ($p = 0.008$; OR = 1.34). Of note, the frequency of the G allele at rs737865 among female cases (0.38) was different than all three comparison groups, namely female controls (0.29), male cases (0.29) and male controls (0.28). Gender-related differences were not consistent with the findings of Shifman *et al*. at the other two SNPs (rs165599 and rs4680).

We next tested epistatic interactions among pairs of SNPs from different genes when a main effect was observed (cutoff set at $p < 0.10$, $n = 22$ SNPs including rs6347 based on stage III, see what follows; total 169 tests). We identified significant interactions between 17 locus pairs ($p \leq 0.05$). Notably, seven of 17 significant interactions (41.2%) involved either rs3756450 in the 5′ upstream region of *SLC6A3* or rs464049 within intron 4 of *SLC6A3* (LD between these SNPs: $r^2 = 0.04/D' = 0.56$). In sum, 29 putative interactions were detected at $p \leq 0.10$.

Table 2. Significant associations from joint analyses of stage I

Chr	Gene	SNP	BP	Stage I families (150 trios)		Stage I case–control ($n = 328/501$)		Stage I joint analysis	
				Z_1	p_1	Z_2	p_2	Z_{joint}	p_{joint}
3	*DRD3*	rs324030	115364131	2.25	0.024	1.48	0.139	2.48	0.013
3	*DRD3*	rs10934256	115368342	2.62	0.009	1.10	0.271	2.36	0.018
3	*DRD3*	rs6280	115373505	2.01	0.044	1.70	0.089	2.52	0.012
3	*DRD3*	rs1800828	115374239	1.97	0.049	1.20	0.23	2.08	0.038
5	*SLC6A3*	rs27072	1447522	3.26	0.001	1.89	0.059	3.37	0.0007
5	*SLC6A3*	rs403636	1491354	3.53	0.0004	2.46	0.014	4.00	0.00006
10	*SLC18A2*	rs3633343	119004938	1.94	0.052	2.09	0.037	2.81	0.005
22	*COMT*	rs737865[a]	18310121	1.31	0.19	2.65	0.008	2.93	0.003
22	*COMT*	rs165815	18334027	1.68	0.09	1.97	0.05	2.55	0.011

Only SNPs associated on the basis of the joint distribution of test statistics ($p_{joint} < 0.05$) are listed.
Z_1, Z_2: Z scores from the analysis of family-based and case–control samples, respectively.
p_1, p_2: Probability of Z score (p-values) from association analyses of family-based and case–control samples, respectively.
Z_{joint}, p_{joint}: Joint analyses and corresponding p-values when considering test statistics and proportion of total samples genotyped in each design.
[a]Analyses in females, conducted on the basis of previous findings by Shifman *et al*. (27).

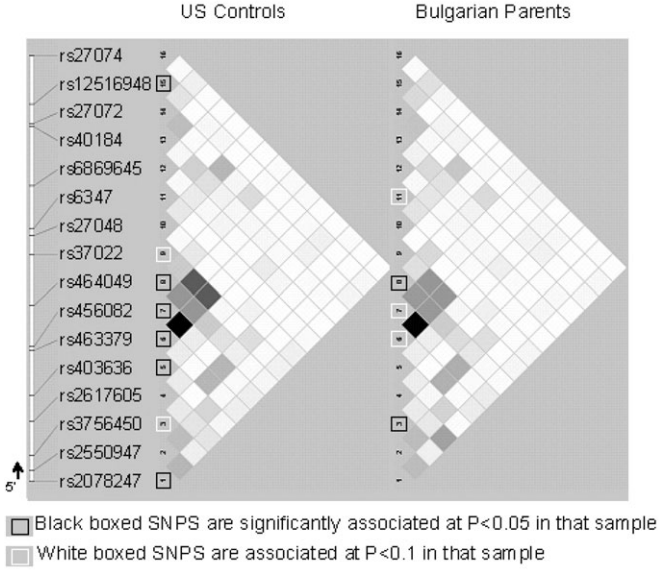

Figure 2. LD among *SLC6A3* SNPs. LD patterns among all *SLC6A3* SNPs genotyped in the US and Bulgarian samples (16 SNPs were common to both samples). LD values between pairs of SNPs (r^2) are indicated, and associated SNPs ($p < 0.10$ and $p < 0.05$) are shown.

Stage III: corroboration with an independent Bulgarian family sample

On the basis of our findings in stages I and II, we tested our hypotheses in a third independent sample composed of 659 case-parent trios from Bulgaria (total $n = 1977$), using 65 SNPs.

Significant associations were again detected in this cohort for both consistently interacting dopamine transporter SNPs in the stage II epistatic analyses (*SLC6A3*: rs464049, $p = 0.011$ and rs3756450, $p = 0.035$). Trends for transmission bias ($p < 0.10$) were detected at five *SLC6A3* SNPs. Associations were not detected with other SNPs, including the three key exonic polymorphisms recently shown to alter COMT mRNA secondary structure (rs4680, rs4633, rs4818). We tested rs737865 for gender-related differences on the basis of our stage II results and again noted significant transmission distortion to female probands ($p = 0.04$, OR = 1.47) but not male probands ($p = 0.18$); however, the overtransmitted allele was the A allele, in contrast to the US samples. The joint distribution of test statistics for SNP analyses from stages II and III (US case–control and Bulgarian trios, respectively) found individual SNP associations at all four genes ($p_{joint} < 0.05$), including seven *SLC6A3* loci (Table 3).

We next tested the putative epistatic interactions from the US sample in this cohort. Interaction tests were limited to the 29 SNP pairs in which epistasis was detected in the US sample at $p \leq 0.10$ or better using a conditional logit model. Remarkably, seven of these 29 interactions (24.1%) were significant ($p \leq 0.05$) in this independent family-based cohort. Consistent with the patterns observed in the US sample, interactions with *SLC6A3* loci were replicated with each of the other three genes (e.g. $p \leq 0.05$ for the same locus pairs in both samples when analyzing *SLC6A3*DRD3, SLC6A3*SLC18A2* and *SLC6A3*COMT*) (Fig. 3). One *DRD3*SLC18A2* interaction was also significant in both samples. Table 4 lists all pairs of loci in which at least a trend ($p \leq 0.10$) was detected in both samples (Table 4).

To interpret the results from our interaction tests, we performed simulations of our analysis design. Using permutation and rejection sampling methods, we emulated the complicated multi-stage design employed here. The simulation results suggest it would be unusual to obtain seven or more 'replicated interactions', such as in stage III. From the simulations, we estimate the probability of this event to be roughly one in a thousand (0.0013 ± 0.00071). Similarly, we estimate the results of finding the initial 29 interaction 'trends' ($p \leq 0.10$) in stage II to also be rare, despite the much larger number of tests (0.0078 ± 0.0055).

Table 3. Associated SNPs at *SLC6A3*, *DRD3*, *SLC18A2* and *COMT*: joint analyses of US and Bulgarian samples

Gene	SNP	Position	N	CEU/JPT/YRI	US Cases/controls (478/501)				Bulgaria trios (659)				Joint analyses	
					Freq	Z_1	p_1	OR	Freq	Z_2	p_2	OR	Z_{joint}	p_{joint}
SLC6A3	rs12516948	1444369	A	0.67/0.81/0.56[a]	0.67	−2.5	0.01	0.79	0.65	−1.3	0.21	0.90	−2.6	0.009
SLC6A3	rs6347	1464412	A	0.72/0.93/0.38	0.71	1.1	0.26	1.12	0.75	1.7	0.10	1.17	2.0	0.046
SLC6A3	rs464049	1476905	C	0.51/0.63/0.74	0.52	2.5	0.01	1.25	0.53	2.5	0.01	1.22	3.5	0.0004
SLC6A3	rs456082	1483515	T	0.70/0.51/0.46	0.77	2.2	0.03	1.27	0.77	1.7	0.09	1.17	2.7	0.007
SLC6A3	rs463379	1484164	C	0.70/NA/0.47	0.77	2.1	0.04	1.26	0.77	1.8	0.07	1.20	2.7	0.006
SLC6A3	rs403636	1491354	G	0.79/0.64/0.78	0.85	−2.0	0.05	1.27	0.85	−1.5	0.15	0.85	−2.4	0.017
SLC6A3	rs3756450	1501148	T	0.84/0.57/0.50	0.87	1.7	0.09	1.27	0.85	2.1	0.04	1.27	2.7	0.007
DRD3	rs7625282	115364217	A	0.73/0.76/0.72	0.76	2.5	0.01	1.26	0.74	0.6	0.52	1.06	2.1	0.033
SLC18A2	rs363393	118995757	A	0.83/1.0/1.0	0.81	1.1	0.28	1.10	0.84	1.9	0.06	1.22	2.1	0.033
SLC18A2	rs363338	118999379	T	0.69/0.24/0.32	0.66	2.2	0.03	1.26	0.67	0.7	0.46	1.06	2.0	0.043
SLC18A2	rs363227	119016556	C	0.89/0.71/0.68	0.87	1.4	0.17	1.15	0.87	1.5	0.13	1.21	2.0	0.041
COMT	rs174696	18327730	T	0.81/0.54/0.34	0.79	2.0	0.05	1.24	0.84	1.3	0.19	1.16	2.3	0.029
COMT	rs165815	18334027	T	0.88/0.65/0.41	0.83	1.8	0.07	1.26	0.78	1.4	0.15	1.15	2.3	0.017

SNPs are listed only if joint distribution of test statistics from stages II and III resulted in $p_{joint} < 0.05$. N, nucleotide. Allele frequency (Freq) of the common allele is given. Allele frequencies from HapMap data for Caucasians (CEU), Asians (JPT) and Africans (YRI) are given. Direction (sign) of the *Z* score is provided for the common allele (e.g. Z = −2.5 indicates that the less common allele confers risk). OR, odds ratio for common allele.
[a]Reference data from Applied Biosystems AoD submission for Caucasian, Japanese and African-American populations (www.ncbi.nlm.nih.gov/SNP).

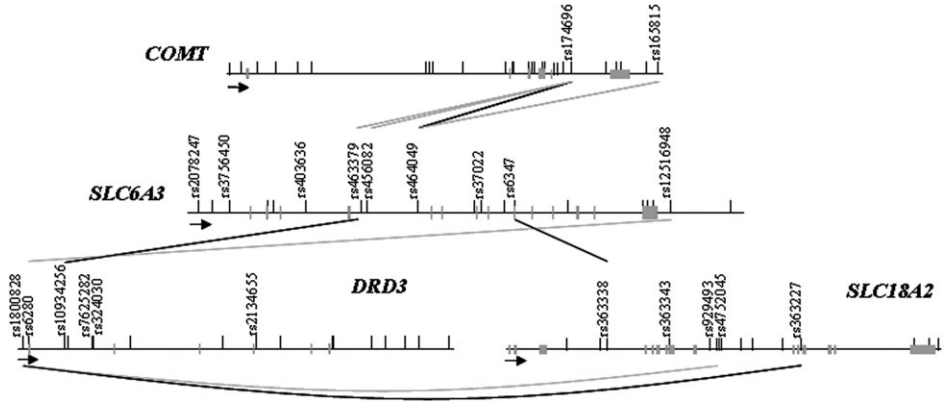

Figure 3. Epistatic interactions at *SLC6A3*, *DRD3, SLC18A2* and *COMT*. The genomic organization of all four genes retained from stage I analyses is shown. Boxes extending below the horizontal line indicate exons, and black tick marks represent all SNPs analyzed in the US and Bulgarian samples. The SNPs retained for epistatic interactions (i.e. SNPs where $p \leq 0.10$ for main effects) are listed. Gray lines indicate epistatic interactions at $p < 0.10$ in both the US and Bulgarian samples; bold black lines indicate significant interactions in the US as well as the Bulgarian samples at $p < 0.05$.

Stage IV: functional analysis

We selected rs3756450 and rs464049 for further analyses of allele-specific functional effects, as these *SLC6A3* SNPs were associated individually with risk for SZ in both samples and featured prominently in the epistatic analyses.

We performed electrophoretic mobility shift assays (EMSA), using nuclear extracts from SHSY-5Y cell line (Fig. 4A). Both allelic probes at rs3756450 generated DNA–protein gel shift bands. Addition of 50-fold unlabeled oligonucleotides probes for each allele inhibited formation of the gel shift bands, demonstrating specificity for these oligonucleotide sequences. We observed three distinct DNA–protein gel shift bands for the T allelic probe at rs3756450. In contrast, the C allelic probe at rs3756450 annealed to only two of the three bands, indicating allele-specific difference in DNA–protein complex formation. The result was replicated in two additional experiments, including one in which 2-fold excess of nuclear extract was added for assays with the C allele (Fig. 4A). In contrast, no allele-specific DNA–protein gel shift bands were observed at rs464049, though bands common to both alleles were noted (data not shown).

Since rs3756450 is localized 5′ to the putative promoter region of *SLC6A3*, we also evaluated its effect on transcription. Dual-luciferase assays were conducted using four clones from CEPH individuals whose genotypes were known (two constructs for each allele, Fig. 4A). Significant promoter activity was present in all constructs, compared with the promoterless construct. In addition, promoter activity was significantly different between constructs carrying C and T alleles (Student's *t*-test, t = 10.32, 5 d.f., $p < 0.0001$; Fig. 4B).

DISCUSSION

Our systematic multi-stage approach yielded novel SNP associations and replicated epistasis between four dopaminergic genes, *SLC6A3*, *DRD3*, *COMT* and *SLC18A2*. We also

Table 4. Noteworthy epistatic interactions at *SLC6A3, DRD3, SLC18A2* and *COMT*

Genes	Loci	Stage II: US case–control		Stage III: Bulgarian trios		Combined results	
		Interaction *p*-value[a]	Perm. *p*-value[b]	LL^{c}_{Diff}	*p*-value	χ^{2d}_4	*p*-value
*SLC6A3*COMT*	rs464049*rs174696	0.005	0.001	5.2	0.023	18.1	0.001
	rs464049*rs165815	0.001	0.001	2.5	0.101	17.7	0.001
	rs463379*rs174696	0.091	0.013	6.7	0.009	14.1	0.007
	rs456082*rs174696	0.069	0.009	5.9	0.015	13.7	0.008
*SLC6A3*SLC18A2*	rs6347*rs363338	0.030	0.023	7.9	0.005	17.6	0.001
*SLC6A3*DRD3*	rs463379*rs10934256	0.047	0.063	5.3	0.021	13.8	0.012
	rs12516948*rs6280	0.099	0.005	3.8	0.052	10.5	0.033
*DRD3*SLC18A2*	rs1800828*rs363227	0.026	0.017	4.4	0.036	13.9	0.008
	rs1800828*rs929493	0.051	0.021	3.4	0.065	11.4	0.022

Results of epistatic interactions between stages II and III. The first column lists the pairs of genes at which interactions were detected. The second column lists the corresponding pairs of SNPs. For example, rs464049*rs174696 denotes an SNP at SLC6A3 interacting with a COMT SNP. Only interactions detected from both samples at $p \leq 0.10$ are listed.

[a]*p*-value for interaction above and beyond main effects in logistic regression (70).

[b]Perm., permutation; *p*-value from 1000 iterations permuting case–control status.

[c]Difference in −2 loglikelihood of full model including an interaction term and a reduced model including only main effects (distributed as a χ^2_1).

[d]Test statistic from combining *p*-values from US and Bulgarian analyses (χ^2_4).

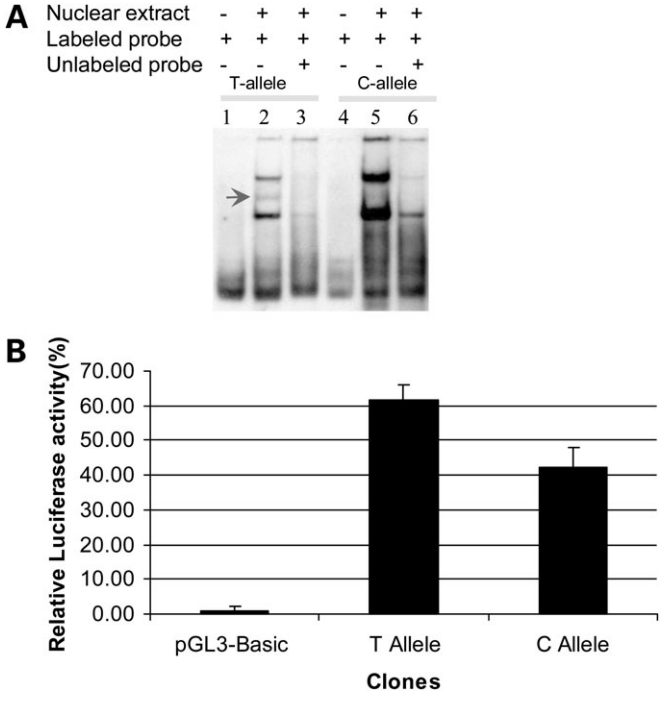

Figure 4. EMSA and promoter assays using rs3756450 at *SLC6A3.* (**A**) Nuclear extracts from SHSY-5Y cells were incubated with labeled probes. The labeled probe for the T allele was loaded in the first three lanes, and the labeled probe for the C allele in the next three lanes. Unlabeled competitor oligonucleotides were included in 50-fold molar excess in lanes 3 and 6. Lanes 1 and 4 indicate the migration of the labeled probe without the nuclear extract. Asterisk indicates altered band shift pattern for T allele of rs3756450 (lane 2) with respect to C allele (lane 5), despite 2-fold excess of nuclear extract added to lanes 5 and 6. (**B**) Promoter activity in a dual-luciferase assay system for constructs containing either the C or T allele at rs3756450 but identical at all other bases.

noted plausible allele-specific functional effects *in vitro* for one of the associated *SLC6A3* SNPs (rs3756450). Three of these genes have been frequent targets in previous SZ association studies (*DRD3, COMT, SLC6A3*), yet prior studies have not provided definitive evidence for or against associations. Overall, the *SLC6A3* associations were most striking. More than a third of test statistics for stage II analyses involving *SLC6A3* SNPs were significant. The median trends test statistic among 18 SNPs was 2.26, indicating a significant shift towards small *p*-values compared with expectations. When we sought evidence for epistasis, SNPs at *SLC6A3* also dominated the list. Two *SLC6A3* SNPs (rs3756450 in the 5′ upstream and rs464049 at intron 4) were involved in 41.2% of the interactions in the US samples. When we evaluated an independent Bulgarian sample, both these SNPs were again associated. Though the dopamine transporter has long been a target for genetic association studies of SZ (reviewed in 28), most reports have focused on a variable number tandem repeat polymorphism localized to 15th exon (29), but meta-analysis does not suggest an association (29). A previous analysis of the *SLC6A3* 3′ VNTR in a subset of the Bulgarian families also was not significant (30). Associations with other *SLC6A3* polymorphisms have been reported, including significant associations in the 5′ region near the promoter (31–33).

At *DRD3*, the present associations are consistent with our previous report, which analyzed a smaller set of US cases and a different group of control samples (20). They follow in a long line of studies that have targeted rs6280, a non-synonymous functional polymorphism (19). More recent studies have shown associations with other variants in both the 5′ and 3′ regions of the gene (16).

A functional exonic SNP (rs4680, Val/Met) has been the focus of numerous association studies at *COMT*, but the results have not been replicated consistently (34–38; reviewed in 39). Associations with haplotypes including rs4680 have been reported recently among Chinese and Ashkenazi Jewish samples (27,40). The latter reported on a haplotype of large effect size comprising three SNPs spanning the gene (rs737865–rs4680–rs165599), and the association was more significant among women. This haplotype was later found to

be associated with decreased COMT mRNA levels in the human brain (41). Gender-specific associations have also been detected with an SNP in this haplotype (rs737865) in late-onset Alzheimer's disease with psychosis (42). Our US samples revealed a gender-related association between SZ and rs737865 consistent with the Shifman results (OR = 1.34). In contrast, our analyses of the Bulgarian sample found overtransmission of the opposite risk allele (A allele), matching the results of Sweet *et al*. (42). Unlike the other three candidates, to date only one small association study of Japanese families at *SLC18A2* has been conducted (43).

The epistatic interactions suggest a susceptibility model in which variations at *SLC6A3* are important determinants of SZ susceptibility, with additional risk due to variants at *SLC18A2*, *DRD3* and *COMT*. This model is appealing because all four proteins regulate synaptic dopamine concentrations, and there are plausible functional relationships between these genes. The dopamine transporter (DAT) controls both the intensity and duration of dopamine actions at synapses by modulating re-uptake into the pre-synaptic nerve terminal (44,45). Because DRD3 may function as an autoreceptor (46,47), it is reasonable to suggest molecular interactions between DRD3 and DAT. Indeed, DRD3 as well as the dopamine D2 (DRD2) receptor subtypes can regulate DAT function (48,49). However, the molecular details of this 'cross-talk' are not known. Since VMAT2, the protein encoded by *SLC18A2*, mediates the transport of dopamine into synaptic vesicles, molecular interactions between VMAT2 and DAT following DAT-mediated re-uptake of dopamine into pre-synaptic terminals are possible and require investigation. Finally, COMT is a key enzyme regulating synaptic dopamine levels through catabolism (50). Common homeostatic mechanisms may thus regulate COMT and DAT.

EMSA analyses suggest specific bandshift patterns using rs464049 probes in neuroblastoma cell lines. More intriguing allele-specific effects were observed with rs3756450, which is localized upstream to the core promoter sequences (51). Our results suggest a putative transcription factor that either has differential affinity for the rs3756450 alleles or binds to rs3756450T but not rs3756450C. Furthermore, luciferase promoter assays suggest significant differences in promoter activity for alleles of this SNP. Thus, sequences flanking rs3756450 may represent a novel promoter domain for *SLC6A3*.

There are some limitations to our association analyses. Though the SNP selection in stage I was more extensive than past studies, more comprehensive coverage would have been desirable for several genes, particularly *MAOA*, *MAOB* and *DDC*. The samples available for stage I analyses were also limited. We estimate only 41.3% power to detect an effect size of 1.5, so type II errors were possible and undetected liability loci could be present at genes that were not carried forward in stage II. Similarly, our family-based US samples had limited power to replicate other reported associations. Our tests of epistasis were relatively conservative, as we considered only locus pairs with evidence of a main effect. Evaluation of much larger samples would be required to conduct an exhaustive analysis of all potential interactions across a larger network of dopaminergic genes.

Our study design was intended to first identify promising susceptibility targets and then test these targets as comprehensively as possible. Spurious associations arising from population substructure are unlikely to account for the *SLC6A3* results, as significant associations were detected in both of the family-based samples. Genomic control (GC) analyses also did not detect meaningful population substructure, and no corrections were necessary. To limit false-positive results, we employed three independent samples, analyzing them independently and jointly. We also simulated our study design and empirically determined the probability of obtaining similar results to the epistatic interactions. These simulations suggest that both our stage II and stage III interaction findings are unlikely under the null hypothesis (about eight in one thousand and one in one thousand for stages II and III, respectively).

In conclusion, our analyses of 18 dopaminergic genes among over 3000 participants indicate that variants at *SLC6A3*, *DRD3*, *COMT* and *SLC18A2* individually and jointly confer risk for SZ. Our findings propose a model for SZ risk in which risk conferred by *SLC6A3* variations could be modified by variants at *DRD3*, *COMT* and/or *SLC18A2*.

MATERIALS AND METHODS

Samples

Unrelated patients from the USA were recruited at Western Psychiatric Institute and Clinic, Pittsburgh, PA, USA and surrounding regions ($n = 478$). Diagnoses were based on the Diagnostic Interview for Genetic Studies (52), supplemented by medical records and informant interviews. Consensus DSM IV diagnoses of SZ ($n = 272$) or schizoaffective disorder (SZA; $n = 206$) were assigned by board-certified psychiatrists/psychologists following review of all these sources of information. Both parents of 150 patients were ascertained for family-based analyses (150 trios). Control DNA samples were collected from the cord blood of 501 unscreened Caucasian neonates born at Magee-Women's Hospital, Pittsburgh, PA, USA. Ancestry and gender were available for all samples.

The Bulgarian patients and their parents were recruited in Bulgaria as part of a collection of parent—proband trios described previously (53). Diagnoses were made according to DSM-IV criteria, following assessment by a psychiatrist using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (54) which has been validated for use in the Bulgarian language by one of the authors of the SCAN instrument, and inspection of hospital discharge summaries. In cases in which the information collected did not allow a confident diagnosis, the patient was re-interviewed by Dr Kirov or the clinical coordinator of the project. All patients and their parents received written information on the project and signed an informed consent form. The Bulgarian sample included 659 trios ($n = 1977$). Probands were diagnosed with SZ ($n = 576$) or SZA ($n = 83$).

The University of Pittsburgh Institutional Review Board approved the study. Written informed consent was obtained from all participants, except neonatal controls, in accordance with IRB guidelines. Approval was obtained from Ethics

Committees in all regions of Bulgaria where families were recruited.

Polymorphism selection

We initially selected SNPs from the Celera database (Celera, 2003), the most comprehensive source available when stage I analyses were initiated. SNPs were selected on the basis of physical distance (1 SNP/5 kb attempted). A denser set of SNPs were then chosen for *SLC6A3*, *SLC18A2*, *DRD3* and *COMT* in stage II. Polymorphisms for each gene were obtained by surveying the genomic region spanning the gene and at least 5 kb of flanking sequence. For *SLC6A3* and *SLC18A2*, SNPs were identified from available HapMap data (HapMap phase I, October 2005 release for *SLC6A3* and HapMap phase II, June 2006 release for *SLC18A2*) (26), and tag SNPs were chosen using Hclust software (55). Hclust computes a similarity matrix from the square of Pearson's correlation (r^2) between allele counts at pairs of loci and then uses hierarchical clustering to group correlated SNPs (minor allele frequency $>5\%$). Tag SNPs were chosen if the correlation between loci was below an arbitrary threshold ($r^2 < 0.8$). One redundant SNP (rs456082) was also chosen. At *COMT* and *DRD3*, additional SNPs were obtained from in-house sequencing. For *DRD3*, we sequenced overlapping 600–800 bp amplicons across the entire gene and 5 kb of flanking sequence in a pool of 200 Caucasian cases to detect common polymorphisms (minor allele frequency $>5\%$). When polymorphisms were detected that were not available in HapMap, we sequenced the same 60 unrelated CEPH individuals used by HapMap to investigate patterns of LD. Sixty-nine polymorphisms were detected, 15 of which were novel, and 18 SNPs were selected (Supplementary Material, Table S2). The 18 SNPs included tag SNPs (chosen with Hclust as discussed earlier), and SNPs associated with SZ in our previous study (20). At *COMT*, 27 SNPs were identified from directly sequencing coding regions and flanking intronic sequence for exons 2–6 as well as the proximal promoter region for S-COMT within intron 3. Sequencing was performed among 60 Caucasian US subjects [data provided by R. Weinshilboum and J.Z., Mayo Clinic, Rochester, MN, USA; (56)]. Individuals used for these analyses were different than those used in HapMap. In sum, we chose 20 SNPs from the combination of HapMap and individual sequencing, realizing redundancy in SNP selection could result. It should be noted that for *COMT*, some SNPs obtained within the predetermined flanking sequence were localized to other genes (*ARVCF* or *TXNRD2*); however, for clarity, these SNPs are still referred to as 'COMT' SNPs. In the Bulgarian sample, 65 SNPs were genotyped. Where possible, identical SNPs to stage II were analyzed ($n = 59$ SNPs). Four additional SNPs were genotyped as surrogates for stage II tag SNPs, and two functional synonymous SNPs at *COMT* were genotyped on the basis of work by Nackley *et al*. (57) during the course of this study (Supplementary Material Table S2).

Since a case–control design was used in stage II, we sought evidence for population substructure by implementing GC analyses using 31 SNPs (58,59). We chose a single common SNP (minor allele $>10\%$) from each of the 31 genomic bins least likely to be linked to SZ from a meta-analysis of linkage scans by Lewis *et al*. (60).

Genotyping assays

Stage I. The screening SNPs ($n = 95$) were assayed using multiplexed polymerase chain reaction (PCR) amplification, followed by single base extension (SNaPshot, ABI Biosystems), as described elsewhere (61).

Stage II. SNPs were genotyped using the hybridization-based Illumina Golden Gate assay, as described elsewhere (62). In sum, 99 SNPs were assayed, including 31 GC SNPs. The median trends test statistic for GC was 0.336 (expected median 0.456), yielding no evidence for noteworthy substructure. Hence corrections were not applied, as it would be anti-conservative (58).

Stage III. Genotyping in the Bulgarian sample was conducted at both Cardiff University (Cardiff, Wales, UK) and the University of Pittsburgh (Pittsburgh, PA, USA). At Cardiff, 33 of the *SLC6A3* and *DRD3* SNPs were genotyped by the Sequenom MassARRAY^{TM} system using iPlexTM chemistries according to the recommendations of the manufacturers (Sequenom, San Diego, CA, USA, http://www.sequenom.com). At Pittsburgh, SNPlex (63) and SNaPshot assays (ABI Biosystems Inc.) were utilized to type the remaining 32 SNPs.

Quality control

In the stage I family-based analyses, we sequenced eight cases for all SNPs (752 sequenced genotypes) and one discrepancy was observed between the sequencing data and the SNaPshot data. In stage I and II case–control analyses, there was complete concordance between Illumina genotypes and HapMap genotypes for 11 CEPH individuals. Among 3024 duplicated genotypes from positive controls, no discrepancies were found. In stage III, we assayed 31 CEPH individuals ($n = 2139$ genotypes) and found five discrepancies. In addition, four SNPs (rs464049, rs463379, rs324030, rs167771) were genotyped in duplicate for all 1977 Bulgarian samples at Pittsburgh and Cardiff (15,816 total genotypes) and 24 discrepancies were found (stage III estimated error rate 0.0015–0.0023). The mean genotype call rate was 99.83% for stage II and 95.71% for stage III.

Mendelian inconsistencies and deviations from Hardy–Weinberg expectations for individual SNPs were evaluated using PEDCHECK (64) and GENEPOP (version 1.31) software, respectively. We detected nine Mendelian errors among the 95 SNPs assayed in stage I and 74 Mendelian errors from analyses of 65 SNPs in 659 Bulgarian trios. In sum, 18 families were removed from Bulgarian analyses owing to multiple Mendelian errors, and remaining sporadic errors were set to null. Hardy–Weinberg equilibrium was tested in each population separately (US cases, US controls, US parents, Bulgarian parents, Bulgarian cases).

Electrophoretic mobility shift assay

Non-radioactive EMSA for rs3756450 was performed using DIG Gel Shift Kit (Roche Applied Science, Inc.) according to the manufacturer's protocol with slight modifications. Polyacrylamide gel electrophoresis-purified 42-base primers (Integrated DNA Technologies, Inc.) encompassing rs3756450 were annealed to complementary oligonucleotides by incubating them at 95°C for 5 min, followed by gradual cooling to room temperature. Annealed double-stranded oligonucleotides were labeled according to the manufacturer's protocol (Roche Applied Science, Inc.). Nuclear extracts were prepared from SHSY-5Y cell lines, as described (65). DIG-labeled oligonucleotides were incubated with nuclear extracts (5 μg) in 20 μl reaction containing 5× binding buffer, poly-L-lysine, poly{d(I-C)}, for 30 min at room temperature. Competitive binding was performed by including 50× unlabeled oligonucleotides in appropriate control reactions. DIG-labeled oligonucleotide–nuclear extract complexes were resolved on 6% non-denaturing polyacrylamide gel for 2 h at room temperature and transferred on positively charged nylon membranes (Boehringer Mannheim–Roche Applied Science, Inc.) by electroblotting. Blots were visualized by an enzyme chemiluminescent method (Roche Applied Science, Inc.). The experiment was replicated, with 2-fold excess of the nuclear extract added to reactions having the rs3756450C probes (Fig. 4A, lanes 4–6). The primer sequences used for generating allele-specific probes are as follows, with altered bases in bold:

rs3756450 T allele FWD: 5′ TAGCAGCAACCACAATGATAA**T**AAAGCCGACTTGGCATTTAG 3′; rs3756450 T allele REV: 5′ CTAAATGCCAAGTCGGCTTT**A**TTATCATTGTGGTTGCTGCTA 3′; rs3756450 C allele FWD: 5′ TAGCAGCAACCACAATGATAA**C**AAAGCCGACTTGGCATTTAG 3′; rs3756450 C allele REV: 5′ CTAAATGCCAAGTCGGCTTT**G**TTATCATTGTGGTTGCTGCTA 3′.

Dual-luciferase assay

A 2.8 kb genomic region encompassing the 5′-UTR of *SLC6A3* (−2783 to +63, spanning rs3756450) was amplified from two CEPH samples homozygous for alleles of rs3756450, using the Expand High Fidelity PCR System (Roche Applied Science, Inc). The PCR-amplified fragments were cloned between *Kpn*1 and *Hin*dIII restriction sites in a pGL3 basic vector (Promega, Inc). Sequence homology for all residues was confirmed by sequencing. Transient transfections of constructs into neuroblastoma cell line SHSY-5Y (ATCC-CRL-2266) were performed in 24-well plates (0.8×10^6 cells/well), using LipofectAMINE (Life Technologies, Inc.), according to the manufacturer's instructions. The pRL-TK (Promega, Inc.) vector expressing Renilla luciferase by an HSV-TK promoter was co-transfected with each construct as an internal control, to normalize for firefly luciferase expression. Cells were harvested 30 h after transfection, and luciferase assays performed using the dual-luciferase reporter assay system (Promega, Inc.). Relative luciferase values were normalized from a promoterless pGL3 BASIC vector. Six readings were taken for each clone, and the entire experiment was conducted in triplicate.

Statistical analysis

Evidence for transmission distortion was assessed using FBAT software (66). Differences in genotype distributions between cases and controls were evaluated with the Armitage trends test (SAS software) (58). Markers localized to the X chromosome (*MAOA* and *MAOB*) were analyzed using likelihood ratio tests in a loglinear model, as implemented in the UNPHASED software suite (67,68). We tested for gender differences at each of the three *COMT* SNPs previously described by Shifman *et al*. (27) (rs4680, rs737865, rs165599) using logistic regression. Gender comparisons were made only for these three SNPs.

To evaluate results from multiple samples, we computed the joint distribution of test statistics (Z_{joint}) on the basis of the methods of Skol *et al*. (25). Here, when combining our results from stages II and III, the proportion of markers genotyped remained the same, and thus Skol *et al*.'s adjustment for variable number of markers genotyped was not applied. *Z*-statistics were derived for both case–control and family-based association tests. To calculate Z_{joint}, let n_1 and n_2 be the sample sizes from which test statistics Z_1 and Z_2 were calculated. The formula for Z_{joint} is then $Z_{\text{joint}} = \sqrt{\pi_1}(Z_1) + \sqrt{1-\pi_1}(Z_2)$, where $\pi_1 = n_1/(n_1 + n_2)$. It should be noted that the sign of the test statistic (i.e. *Z* positive or negative) was accounted for in all analyses, meaning the risk allele was required to be the same in both samples. To determine π_1, or the proportion of total samples genotyped in the first stage, we treated a complete case-parent trio ($n =$ 3 individuals) as the equivalent of one case and one control. For stage I, we therefore had the equivalent of 478 cases and 651 controls available, of which the 150 trios represented 26.6% of these samples (i.e. $\pi_{\text{samples}} = 0.266$). In this staged design, we calculated Z_{joint} twice, once over the case–control and family-based association analyses in stage I (US samples only), and again over the case–control analyses of stage II and the family-based association analyses of stage III (US and Bulgarian samples). Where surrogates were chosen in the Bulgarian sample to represent tag SNPs in the US sample, Z_{joint} was calculated by combining the test statistic from the original SNP with that of the surrogate. In using this procedure, we are confident that the size of the test was not likely to be altered (e.g. $p = 0.05$ was still at least a 5% type I error threshold). However, a lower correlation between SNPs could result in loss of power.

Epistatic interactions were tested in stages II and III. Pairs of loci, each of which provided a *p*-value <0.10 for a main effect on risk for SZ, were analyzed for interaction effects using an unconditional logit model for case–control analyses and a conditional logit model for trios (69). In both instances, the 'interaction *p*-value' reported represented the likelihood difference between a full model including both main effects and an interaction term from a reduced model including only main effects. When interaction results were significant by asymptotic approximation, empirical *p*-values were determined by permutation testing (1000 permutations; Genetic Association and Interaction Analysis software) (70).

For the functional analyses, we used a paired *t*-test to determine differences in luciferase activity between the C and T

alleles at rs3756450. To determine significant differences between constructs, we conducted analysis of variance.

Simulations

Interpretation of the interaction results from the staged design is complicated by the design itself and by the LD structure of SNPs in each gene. To facilitate interpretation of results from interaction tests, we performed a simulation experiment on the basis of the data from these cases and controls. Each simulation consisted of three stages:

Stage I: Permute the case–control status, thus making affection status independent of genotypes while retaining the LD structure of the sample. Test all 68 SNPs individually for association with affection status at two levels of significance ($p \leq 0.05$ and $p \leq 0.10$). If eight or more SNPs are associated at $p \leq 0.05$, then record all S SNPs with $p \leq 0.10$ and proceed to stage II; else reject this set of data and re-run the permutation until eight or more SNPs are associated at $p \leq 0.05$. Rejection sampling ensures that this stage is comparable with the results obtained in the original experiment in terms of the number of SNPs associated with affection status.

Stage II: Using the stage 1 data set and the list of S SNPs, test for all possible SNP–SNP interactions, with the condition that each of the two SNPs be in different genes (i.e. gene–gene interaction). As per the original experiment, record all I interactions having a $p \leq 0.10$ for association.

Stage III: Do a new permutation of case–control data. With these data, test the I interactions found in Stage II, using a significance level of $p \leq 0.05$. Record the number of 'replicated interactions' R.

We performed this stage 1–3 experiment 10 000 times to obtain the distribution of R. This is, in essence, the design of the original experiment. It differs in the sense that the original experiment used a family-based sample in stage III and had slightly different sample sizes, but neither of these features should be important under the null hypothesis evaluated here.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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