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Association and linkage analyses of *RGS4* polymorphisms in schizophrenia

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Gene expression analyses of postmortem cerebral cortex suggest that transcription of the regulator of G-protein signaling 4 (*RGS4*) is decreased in a diagnosis-specific manner in subjects with schizophrenia. To evaluate the possible role of *RGS4* in the pathogenesis of schizophrenia, we conducted genetic association and linkage studies using samples ascertained independently in Pittsburgh and New Delhi and by the NIMH Collaborative Genetics Initiative. Using the transmission disequilibrium test, significant transmission distortion was observed in the Pittsburgh and NIMH samples. Among single-nucleotide polymorphisms (SNPs) spanning approximately 300 kb, significant associations involved four SNPs localized to a 10 kb region at *RGS4*, but the associated haplotypes differed. A trend for transmission distortion was also present in the Indian sample for haplotypes incorporating the same SNPs. Consistent with the linkage/association observed from the family-based tests, samples with affected siblings (NIMH, India) showed higher levels of allele sharing, identical by descent, at *RGS4*. When the US patients were contrasted to two population-based control samples, however, no significant differences were observed. To check the specificity of the transmission bias, we therefore examined US families with bipolar I disorder (BD1) probands. This sample also showed a trend for transmission distortion, and differed significantly from the population-based controls for the four-SNP haplotypes tested in the other samples. The transmission distortion is unlikely to be due to chance, but its mechanism and specificity require further study. Our results illustrate the potential power of combining gene expression profiling and genomic analyses to identify susceptibility genes for genetically complex disorders.

INTRODUCTION

Heritability estimates for schizophrenia range from 70% to 80% (1). However, efforts to map susceptibility genes using traditional linkage studies have been inconsistent (2), probably because of complex patterns of inheritance and the relatively small risk conferred by individual genes (3). An alternative to linkage analyses is to test candidate genes for linkage and association using the transmission disequilibrium Test (TDT) (4). Identification of such candidates has come more rapidly with the use of modern molecular methods, such as gene microarrays (5).

We recently demonstrated that the expression of RGS4, but not other RGS family members, is decreased across the cerebral cortex in subjects with schizophrenia, but not among subjects with major depressive disorder (6). RGS proteins function primarily as GTPase-activating proteins (GAPs) for heterotrimeric G-protein α (G_α) subunits, accelerating the hydrolysis of G_α-bound GTP (7). Thus, they shorten the duration of intracellular signaling of many G-protein-coupled receptors (GPCRs) belonging to dopamine, GABA, glutamate and other neurotransmitter systems. RGS4 is an interesting candidate gene for additional reasons. It is highly expressed in brain regions implicated in the pathophysiology of schizophrenia, modulates

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function of multiple G-protein-coupled neurotransmitter receptors and exhibits robust transcriptional changes to stress (8). Furthermore, RGS4 maps to chromosome 1q21–q22, a region implicated in prior schizophrenia linkage studies (9).

To determine whether altered RGS4 expression reflects a primary inherited abnormality in schizophrenia, we conducted genetic association studies using RGS4 polymorphisms. Genetic associations in schizophrenia have been inconsistent. Some of the reported associations may have been due to population substructure and the limited power of the samples tested (2,3). Therefore, we elected to use combined linkage/association strategies in addition to traditional case–control analyses. To bolster inferences from our relatively small Pittsburgh samples, we also evaluated independent samples ascertained at New Delhi, as well as the NIMH Collaborative Genetics Initiative (10).

RESULTS

SNP selection

We reconfirmed the expression of RGS4 in human cerebral cortex and identified 26 single-nucleotide polymorphisms (SNPs) after resequencing all exons, introns and flanking 5′ and 3′ UTRs (Fig. 1). As described in Materials and Methods, we selected 13 SNPs for analysis using the TDT (4).

Analysis of schizophrenia samples

We employed GENEHUNTER software for TDT analyses of individual SNPs and haplotypes (11). The computer program TRANSMIT was used for global tests of association involving multiple haplotypes (12,13).

Pittsburgh sample. When the SNPs were tested individually by TDT, significantly increased transmission at SNP 4 was observed in the Pittsburgh sample ($P = 0.05$, 1 df). 'Moving-window' haplotype analyses using two to four contiguous SNPs were then conducted to estimate how the association varied across the RGS4 locus and surrounding region (14,15). They revealed significant association for several haplotypes; all but one included combinations of SNPs 1, 4, 7 or 18. These SNPs are localized to the upstream sequence or first intron of RGS4 (Fig. 1, Table 1).

The TDT analyses described above involved individual SNPs or haplotypes, which were contrasted against all the others. To gain an understanding of overall transmission distortion, a global test of association for all haplotypes encompassing SNPs 1, 4, 7 and 18 was conducted. This analysis suggested significant transmission distortion and was subsequently used as the principal method for evaluating results from different samples (TRANSMIT program using 93 case–parent trios, $\chi^2 = 16.6$, 8 df, $P = 0.035$) (12,13).

There were 55 Caucasian cases with schizophrenia in the Pittsburgh sample; the transmission distortion remained significant when the sample was restricted to such individuals ($\chi^2 = 13.0$, 6 df, $P = 0.043$). In the smaller subgroup with schizoaffective disorder ($n = 38$), there was overtransmission of the same haplotype that was associated with schizophrenia, but the global test of association was not significant (TRANSMIT analysis; $\chi^2 = 8.17$, 8 df, $P = 0.417$).

NIMH sample. The SNPs used in the Pittsburgh sample were next analyzed in the ethnically diverse NIMH sample. A global test for association was significant for haplotypes encompassing SNPs 1, 4, 7 and 18 ($\chi^2 = 18.8$, $P = 0.016$, 8 df). Significant transmission distortion was also observed individually at SNPs 1, 4 and 18 ($P = 0.01$, 0.003 and 0.005, respectively, 1 df; Table 1). Exclusion of African–American families from the sample also revealed significant results for each of these SNPs ($P = 0.023$, 0.011 and 0.033, respectively). Moving-window haplotype analyses revealed preferential transmission for more extensive chromosomal segments than the Pittsburgh sample. Like the Pittsburgh sample, all but one of the haplotypes with significant increased transmission included SNPs 1, 4, 7 or 18. However, the transmitted alleles and haplotypes differed from the Pittsburgh sample.

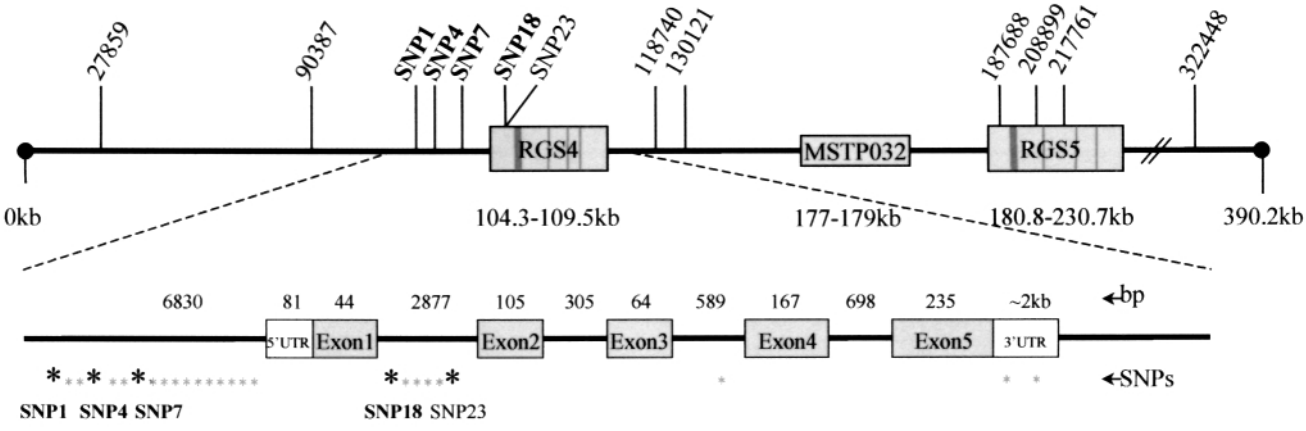

Figure 1. Genomic organization of RGS4 and flanking regions. The genomic organization is derived from available sequences for clone NT_022030, as well as our sequence analyses and is up to date as of January 2002. Five exons were identified from the coding sequence for RGS4 (estimated length for exons and introns 8.5 kb). The SNPs analyzed in the present study are listed in the top panel. *, SNPs identified by resequencing RGS4; *, identified SNPs used for TDT analysis.

Table 1. SNPs and haplotypes at RGS4 with significant preferential transmission

Pittsburgh schizophrenia families			NIMH families		
SNPs	Transmitted allele	T/NT	SNP	Transmitted allele	T/NT
SNP 1	○	53/35 (0.055)	SNP 1	●	30/13 (0.01)
SNP 4	●	51/33 (0.05)	SNP 4	○	22/6 (0.003)
			SNP 18	○	24/8 (0.005)
Haplotypes (SNPs: 27859—90387—1—4—7—18—23—118740—130121—187688—208899—217761—322448)					
/—●—○—●—/—/—/—/—/—/—/—/—/		34/18 (0.03)	○—●—●—/—/—/—/—/—/—/—/—/		9/1 (0.02)
/—●—○—●—○—/—/—/—/—/—/—/—/		33/14 (0.006)	○—●—●—○—/—/—/—/—/—/—/—/		8/0 (0.005)
			/—●—●—○—/—/—/—/—/—/—/—/		11/3 (0.04)
/—/—○—●—/—/—/—/—/—/—/—/		38/17 (0.005)	/—/—●—○—/—/—/—/—/—/—/—/		23/3 (0.0001)
/—/—○—●—○—/—/—/—/—/—/—/		37/13 (0.0007)	/—/—●—○—●—/—/—/—/—/—/—/		19/3 (0.001)
/—/—○—●—○—●—/—/—/—/—/—/		37/13 (0.0007)	/—/—●—○—●—○—/—/—/—/—/—/		19/3 (0.001)[a]
/—/—/—●—○—/—/—/—/—/—/—/		39/19 (0.009)	/—/—/—○—●—/—/—/—/—/—/—/		20/7 (0.02)
/—/—/—●—○—●—/—/—/—/—/—/		39/19 (0.009)	/—/—/—○—●—○—/—/—/—/—/—/		20/7 (0.02)
/—/—/—●—○—●—●—/—/—/—/—/		35/19 (0.03)	/—/—/—○—●—○—●—/—/—/—/—/		11/3 (0.04)
/—/—/—/—○—●—/—/—/—/—/—/		40/22 (0.022)	/—/—/—/—●—○—/—/—/—/—/—/		20/7 (0.02)
			/—/—/—/—●—○—●—/—/—/—/—/		11/3 (0.04)
			/—/—/—/—/—○—●—/—/—/—/—/		20/6 (0.006)
			/—/—/—/—/—○—●—●—/—/—/—/		20/4 (0.001)
			/—○—●—/—/—/—/—/—/—/—/—/		11/3 (0.04)
			/—○—●—○—/—/—/—/—/—/—/—/		11/3 (0.04)
			/—/—●—○—○—/—/—/—/—/—/—/		4/0 (0.05)
			/—/—●—○—○—○—/—/—/—/—/—/		4/0 (0.05)
			/—/—/—/—○—○—●—●—/—/—/—/		7/1 (0.04)
/—/—/—/—/—/—/—○—●—○—○—/—/		6/0 (0.01)	/—/—/—/—/—/—/—/—/—○—●—○—●		5/0 (0.03)

Only statistically significant increased transmissions to cases are shown and have been ordered for clarity. Numbers in parenthesis indicate P-values.
T/NT, Alleles or haplotypes transmitted / not transmitted; open circles, allele 1, filled circles, allele 2 at each locus; '/', allele not specified at this locus.
[a]A trend for increased transmission of this haplotype was also observed in the Indian sample ($P = 0.055$).

Indian sample. Since the US samples indicated associations restricted to SNPs 1, 4, 7 or 18, we investigated these SNPs in a third independently ascertained sample of Indian ethnicity. Globally, a trend for overall transmission distortion for all haplotypes involving these SNPs was observed ($\chi^2 = 22.04$, $P = 0.078$, 14 df). Significant transmission distortion for individual SNPs or haplotypes was not observed. However, we noted a trend for excess transmission of one of the haplotypes associated with the NIMH sample ($\chi^2 = 3.66$, $P = 0.055$, 1 df; see Table 1).

Identity by descent (IBD) sharing. All analyses described thus far involved one case per family. If the significant TDT results were due to linkage, we reasoned that affected sibships should also yield evidence for linkage. For 30 available affected sib-pairs in the NIMH sample, the proportion of 0, 1 or 2 haplotypes identical by descent were elevated over expectations of 0.25, 0.50 and 0.25, namely 0.11, 0.44 and 0.45, respectively [mean sharing 67% for SNPs 1, 4, 7 and 18 analyzed in conjunction with five flanking short tandem repeat polymorphisms genotyped previously (10,16)]. The mean sharing was also elevated, albeit to a lesser degree, among 72 Indian families with sib-pairs (IBD 0.26, 0.44, 0.30 for 0, 1 or 2 haplotypes; mean sharing 52%).

Population-based association analyses. We also tested for association at the population level by comparing Caucasian cases from each US sample separately with two independent groups of unscreened US Caucasian community-based controls, composed respectively of neonates and adults. We analyzed genotypes and allele frequencies for SNPs 1, 4, 7 and 18, the markers that appeared to be relevant following TDT analysis of the schizophrenia samples. We also estimated and compared haplotypes frequencies using SNP-EM, an expectation-maximization algorithm (EM) (17,18) (Table 2). SNP 14 was informative only among African–Americans, and so was analyzed separately using 70 African–American cases and 93 control individuals from Pittsburgh (data not shown). Significant case–control differences were not noted for any of the comparisons.

Analysis of bipolar I disorder (BD1) samples

To evaluate the specificity of the results from the schizophrenia samples, we investigated SNPs 1, 4, 7, 18 and 23 among 101 cases with BD1 and their parents. Like the Indian schizophrenia sample, trends for global transmission distortion of haplotypes were observed using SNPs 1, 4, 7 and 18 (TRANSMIT program; $\chi^2 = 16.99$, $P = 0.108$, 11 df). Significant excess transmission of individual SNPs or haplotypes was not observed, although some SNPs and haplotypes were significantly undertransmitted.

When haplotypes incorporating the above SNPs were compared between the BD1 cases and the neonatal or adult controls, significant differences were observed overall (SNP-EM Omnibus likelihood ratio test; $P = 0.0002$, both comparisons). The associated haplotypes differed from those

Table 2. Estimated haplotype frequencies among cases and unrelated controls

Haplotype	Pittsburgh schizophrenia cases	NIMH cases	Indian cases	Pittsburgh bipolar I disorder cases	Neonatal controls	Adult controls
SNP 1—4—7—18						
○—○—○—○	0.078	0.067	0.141	0.044	0.096	0.066
●—○—○—○	0.022	0.083	0.034	0.029	0.004	0.021
○—●—○—○	0	0	0.033	0	0.006	0.006
●—●—○—○	0	0	0.003	0	0	0
○—○—●—○	0.006	0	0.026	0.025	0	0
●—○—●—○	0.378	0.392	0.426	0.378	0.388	0.442
○—●—●—○	0	0	0	0	0	0.006
●—●—●—○	0.006	0	0.012	0	0	0
○—○—○—●	0	0.017	0.030	0.017	0	0.004
●—○—○—●	0	0	0.003	0	0	0.006
○—●—○—●	0.494	0.417	0.249	0.349	0.439	0.425
●—●—○—●	0	0	0.010	0.007	0.008	0.013
○—○—●—●	0	0	0.002	0	0	0
●—○—●—●	0.017	0.025	0.028	0.050	0.053	0.013
○—●—●—●	0	0	0.003	0.102[a]	0.006	0.000
●—●—●—●	0	0	0	0	0	0

The Caucasian cases from Pittsburgh (schizophrenia, n = 93; bipolar I disorder, n = 82) and NIMH (n = 25) were compared separately with unscreened Caucasian neonatal or adult controls from Pittsburgh (n = 85 and 89, respectively). Frequencies for Indian cases are included for comparison. An omnibus test based on likelihood ratios detected overall differences in haplotype frequencies between the bipolar I cases and each group of controls (P = 0.0002) (18).
For each SNP, open circles represent allele 1 and filled circles allele 2.
[a]Significantly different from adult controls ($P < 0.002$) or neonatal controls ($P < 0.02$) following corrections for multiple comparisons.

observed for the US schizophrenia TDT analyses (Table 2). Significant differences were also present for both sets of comparisons when haplotypes based on two or three adjacent SNPs were analyzed from among SNPs 4, 7, 18 and 23, although significant case–control differences were not observed when SNPs were analyzed individually (data not shown).

Synthesis of TDT results

To synthesize these results, we evaluated the overall probability for biased parental transmission of haplotypes bearing SNPs 1, 4, 7 and 18. Using the TRANSMIT global test of association, the individual P-values were 0.035, 0.016, 0.078 and 0.108, respectively, in the Pittsburgh, NIMH, Indian and BD1 samples. Under the null hypothesis of no association, P-values follow a uniform distribution on the interval (0,1). When transformed by taking twice the negative logarithm, the P-values follow a χ^2 distribution with two degrees of freedom (df). The sum of N independent, transformed P-values follows a χ^2 with 2N df. Accumulating over studies, the overall P-values were thus as follows: for the Pittsburgh schizophrenia sample, 0.035; for the Pittsburgh plus NIMH samples, 0.0048; for the Pittsburgh plus NIMH plus Indian samples, 0.0027; and for all schizophrenia samples plus the BD1 sample, 0.0019.

Variations in levels of linkage disequilibrium (LD)

We evaluated patterns of LD between pairs of SNPs 1, 4, 7 and 18 to see if there are substantial differences in LD among the samples. We used D′ to estimate LD (19). Significant LD was observed for all pairwise calculations among each set of controls, the three sets of cases, and the respective parents of the cases ($D' > 0.78$; $P < 0.00013$ for all comparisons; Table 3). In general, LD between SNPs 7 and 18 was lower than the D′-values for the other comparisons. Across samples, the patterns of LD were remarkably similar, considering the substantial variability of LD. The Indian samples tend to have lower LD and there is an intriguing tendency toward increased LD in the case versus the control samples.

Power analysis

The test statistics for transmission of haplotypes suggest that the transmission bias cannot be very large. Investigations of power in this setting are complicated by two unknowns, namely which haplotypes carry the disease allele(s) and what are their individual effects on risk, as well as ambiguity regarding haplotype identity and transmission. Despite the complications, however, we can provide a rough guide to the sample size required to replicate the current findings by using the realized test statistics and the number of TDT trios assessed to calculate the average per-family contribution to the non-centrality parameter of the χ^2 statistic. Using this approach and the results from the NIMH sample, 69 parent–child trios would be required to replicate with 80% power and a critical P-value of 0.05. The results from the Pittsburgh sample, however, suggest that a larger sample of 130 trios will be required. That latter seems more realistic, and may itself be a substantial underestimate. Seltman et al. (20) have previously published a power study in settings relevant to our present results.

DISCUSSION

With over 1400 subjects and a complementary resequencing effort, our study represents a rigorous examination of a plausible candidate for heritable association with schizophre-

Table 3. Pair-wise LD among cases, parents and unrelated controls

SNP pairs	Pittsburgh schizophrenia cases		NIMH cases		Indian cases		Pittsburgh bipolar I disorder cases		Neonatal controls	Adult controls
	Cases	Parents	Cases	Parents	Cases	Parents	Cases	Parents		
1&4	0.972	1	1	1	0.913	0.877	0.971	0.951	0.969	0.948
1&7	0.976	0.962	1	1	0.893	0.880	0.846	0.863	0.977	0.976
1&18	0.918	0.870	0.901	0.891	0.792	0.775	0.753	0.78	0.786	0.866
4&7	0.970	0.953	1	1	0.935	0.921	0.620	0.537	0.969	0.971
4&18	0.978	1	1	1	0.820	0.796	1	0.959	0.973	0.953
7&18	0.911	0.808	0.870	0.878	0.813	0.867	0.646	0.611	0.780	0.942
Average	0.954	0.932	0.962	0.962	0.861	0.853	0.806	0.784	0.909	0.943

LD was estimated using D′ (19). Significant LD was observed for all pair-wise calculations ($D' > 0.78$; $P < 0.00013$).

nia. When a genomic region of approximately 300 kb was surveyed, associations were detected for four SNPs localized to a 10 kb span at RGS4. Hence, we investigated associations for these SNPs and their haplotypes in all our samples. The global association tests for haplotypes bearing all four SNPs suggest that the transmission distortion is unlikely to be due to chance alone, especially when all family-based samples are considered. It is possible that the TDT results reflect a primary association with another gene in this region, but no significant associations with SNPs flanking RGS4 were detected (Fig. 1, Table 1). In a recent genome-wide survey, significant linkage disequilibrium (LD) typically extended 60 kb from SNPs with common alleles (range 6–155 kb) (21).

The overall probability of the global association using haplotypes for the four relevant SNPs was 0.0027 when the schizophrenia family-based samples were considered. The differing alleles and haplotypes associated with the Pittsburgh and NIMH samples are difficult to reconcile, because both samples are US cohorts. The results, however are not completely inconsistent with the possibility that RGS4 polymorphisms/mutations confer liability. For example, when testing haplotypes for a locus conferring modest liability, Seltman et al. (20) showed that erratic patterns of associated haplotypes can result from significance tests that contrast transmission of individual haplotypes against transmission for all other haplotypes, as we have done for some of our significance tests. Thus, the fact that different haplotypes are significantly associated with schizophrenia liability in the Pittsburgh and NIMH samples, and to a lesser extent in the Indian sample, could be due to the inherent statistical difficulty of identifying associated haplotypes. The differing results could also reflect inherent complexity in the mechanism by which susceptibility is conferred at this locus. In model organisms, for example, very complex genotype–phenotype relationships are now commonly observed (e.g. loci influencing bristle length in Drosophila) (22). Our current approach to an understanding of genotype–phenotype relationships in humans may be inadequate for this level of complexity.

Our TDT results are supported by the increased sharing IBD at RGS4 observed among the affected sib-pairs from the NIMH sample. Further IBD analysis of other affected individuals in multiplex kindreds such as the NIMH sample would be helpful, using microsatellite markers flanking the RGS4 locus as well as the markers used in the present study. Susceptibility haplotypes might be identified by joint analysis of linkage and association in such samples, possibly in conjunction with a control sample (23).

It is also possible that the differing associations in the Pittsburgh and NIMH samples represent independent risk factors. Credible associations with more than one haplotype have been reported for other genetically complex diseases (e.g. psoriasis and asthma/atopy) (15,24). The possibility of two susceptibility alleles (or varying combinations of unidentified nucleotide substitutions) residing on two distinct and common haplotypes, with variable penetrance, therefore can not be discounted. A counter-argument is the observation that associations were not detected when the Pittsburgh or NIMH cases were compared with the population-based controls. Still, the failure to detect association may reflect the reduced power for case–control analyses in the presence of population substructure (25,26), or it may simply reflect the different sensitivities of the tests (27).

Our analyses do not indicate which, if any, of the associated SNPs or haplotypes are directly related to pathogenesis, nor do they clarify the relationship between the genetic associations and our earlier postmortem expression analyses. All RGS4 exons were sequenced among NIMH cases homozygous for the associated haplotype (SNPs 1–4–7–18) and postmortem samples from the prior study (1), but no polymorphisms were detected. The postmortem studies demonstrate altered expression of the RGS4 transcript. Therefore, if RGS4 were to affect liability to schizophrenia, the upstream sequence, which typically regulates gene expression, would be a logical region to examine. Indeed, this is precisely the region in which significant linkage/associations between RGS4 polymorphisms and schizophrenia were detected.

The four-SNP haplotype with significant transmission distortion in the NIMH family-based sample was also transmitted with increased frequency to the ethnically distinct Indian cases. This result and the global test of association using TRANSMIT for haplotypes were only marginally significant when viewed alone. Yet, in light of the other results, the marginally significant results become more compelling. Even so, the effect of polymorphisms at RGS4 on liability to schizophrenia, if any, appears to be small for the Indian sample. Such a result would be unremarkable given the geographical and ethnic features of the populations from which the samples were obtained.

It is well known that significant results from family-based tests such as the TDT can arise from two genetic phenomena: true linkage to liability alleles or general transmission distortion in the vicinity of the tested polymorphisms, such as meiotic drive, which is unrelated to liability. To evaluate the possibility of general transmission bias, we also analyzed a BD1 sample that was available to our group. This sample, while convenient, is not ideal because of the possibility that schizophrenia and BD1 share some sources of genetic liability (28,29). In addition, the BD1 sample lacked power comparable to that of the schizophrenia samples, since the majority of BD1 families had only one available parent (30). Despite this constraint, we observed a trend for transmission distortion when the four-SNP haplotypes were analyzed – a finding that may be consistent with the possibility of a general transmission bias in the chromosome 1q21–q22 region. However, the substantial differences observed between the BD1 cases and both control samples in the distribution of haplotypes weighs against this interpretation. Moreover, there are no known chromosomal structures in this region likely to generate such a bias. We suspect that a stringent test of general transmission bias would require analyses of hundreds of population-based families, since the distortion, if it exists, must be quite subtle and highly variable.

An alternative hypothesis to general transmission bias incorporates a biological link across the psychiatric disorders that comprised our samples. For example, life stressors influence the appearance and recurrence of psychotic symptoms in both schizophrenia and BD1 (31–33). This is particularly interesting in light of recent data demonstrating that RGS4 expression in different brain areas was highly responsive to an experimental model of acute stress (8). Given the central role that RGS4 plays in regulating the duration of postsynaptic signaling for Gi/o and Gq-coupled neurotransmitter receptors (7), the stress-related response in animals may reflect a role for RGS4 in the adaptation of particular brain circuits to stress. In our previous gene microarray studies, we found that RGS4 was the most consistently changed transcript, decreasing by almost 35% in the cerebral cortex of subjects with schizophrenia. Yet we failed to detect a change in expression in samples from subjects with major depressive disorder (6), suggesting that disruption of RGS4 may not be a common factor across all psychiatric disorders. This hypothesis could be tested critically by TDT analyses among probands with recurrent major depressive disorder who are screened for BD1. We suggest that the convergence of data from population-based gene inheritance and expression studies provides a basis for identifying new candidates that may convey susceptibility for specific psychiatric disorders.

MATERIALS AND METHODS

Clinical

Pittsburgh schizophrenia cases and parents. Caucasian inpatients and outpatients with schizophrenia ($n = 55$) or schizoaffective disorder ($n = 38$) were recruited at Western Psychiatric Institute and Clinic, a University-affiliated tertiary care center, and 35 other treatment facilities within a 500-mile radius of Pittsburgh. The Diagnostic Interview for Genetic Studies (DIGS) was the primary source for clinical information for probands (34). Additional information was obtained from available medical records and appropriate relatives. Consensus diagnoses were established by board-certified psychiatrists. An additional group of 70 African–American cases were used exclusively for analysis of SNP14. Genomic DNA was obtained from all parents of the Caucasian cases. One parent was diagnosed with schizophrenia.

Pittsburgh population based control individuals

(i) Neonatal cord blood samples were obtained from live births at Magee–Women's Hospital, Pittsburgh and served as unscreened, population-based controls. There were 169 individuals (85 Caucasian individuals and 93 African–Americans). The African–American control individuals were used only for analysis of SNP14.
(ii) Adults of Caucasian ethnicity were selected from the local community as well as staff members at the University of Pittsburgh ($n = 89$). Though formal psychiatric examination was not conducted, none of them were diagnosed with schizophrenia/schizoaffective disorder.

Pittsburgh bipolar I disorder cases (DSM IV) and parents. Caucasian inpatients and outpatients were ascertained and diagnosed in a manner identical to the schizophrenia families, with the exception that case–parent 'duos' were included. There were 101 cases, of whom 39 had both parents and 62 had one available parent.

National Institute of Mental Health Collaborative Genetics Initiative (NIMH CGI) sample. From 1991 to 1998, pedigrees having probands with schizophrenia or schizoaffective disorder, depressed (DSM IV criteria) were ascertained at Columbia University, Harvard University and Washington University. The DIGS was the primary interview schedule. The families were ascertained if they included two or more affected first-degree relatives (10). We selected case–parent trios, and available affected siblings from this cohort. Thus, DNA samples from 39 cases, their parents and 30 affected sib-pairs were obtained. They comprised 25 families of Caucasian ethnicity, 10 of African–American ethnicity and 4 from other ethnic groups. TDT analysis utilized only one case/family. Thirteen parents were affected.

Indian schizophrenia cases and parents. Using interview and diagnostic procedures identical to that of the Pittsburgh studies, patients were ascertained from psychiatric treatment facilities in the metropolitan New Delhi area. We used the Hindi version of the DIGS (35). Inter-rater reliability was tested between Indian and US psychiatrists throughout the recruitment period, and was consistently high ($\kappa > 0.80$). Population-based controls were unavailable. Apart from proband-parent trios, affected sibs were also recruited. A total of 269 case–parent trio and 72 affected sib-pair families were analyzed.

Written informed consent was obtained from all participants. The University of Pittsburgh Institutional Review Board did not require informed consent from neonatal control individuals, since they were anonymous. Ethnicity was based on self-report (maternal report for neonatal samples).

Laboratory

RGS4 transcript analysis. A Human Multiple Tissue Northern Blot (Clontech) and a ^{32}P-labeled cDNA probe were used to confirm the size of the RGS4 transcript reported previously (36). We noted the presence of single dark bands of approximately 3 kb in lanes from multiple brain regions (whole cerebral cortex, frontal pole, occipital pole and temporal lobe), with much fainter or absent bands being observed in lanes from other brain regions (cerebellum, medulla, spinal cord and putamen). Because the UniGene entry for the RGS4 cDNA (U27768) contains only the truncated coding region (800 bp), we designed custom PCR primers based on the BAC clone sequence containing the RGS4 gene (NT_022030) to obtain the complete RGS4 transcript sequence. For this analysis, mRNA from a control human brain was purified, digested with DNase, and repurified prior to first-strand cDNA synthesis using Superscript II (Gibco) with an oligo dT primer. The resulting cDNA–mRNA mixture was diluted and used in a standard PCR reaction with AmpliTaq Gold (see above). All reaction products yielded single bright bands on 2% agarose/ethidium bromide-stained gels, and were subsequently purified and sequenced. Alignment of these sequences produced over 99% identity matches with the BAC clone sequence containing RGS4. The 3′-UTR for RGS4 obtained in this manner also aligned over 99% with a cDNA entry (AL137433.1) that contains both a poly(A) signal and a poly(A) attachment site, confirming that the human RGS4 transcript is 2949 bp without the poly(A) tail and includes a cDNA entry not previously associated with the human transcript in the NCBI database (Fig. 1).

DNA sequencing and polymorphism detection. The genomic sequences for RGS4 was obtained from NT_022030 (390 242 bp), a currently unfinished clone from the Human Genome Project, Chromosome 1 database. The annotated data revealed three identified genes, namely, RGS4, MSTP032 and RGS5.

A panel of 10 African–American cases and 6 Caucasian controls was initially used to screen for polymorphisms in the exonic, intronic and flanking genomic sequences of RGS4. The resequenced region included 6.8 kb upstream and 2.9 kb downstream of the coding sequence. The genomic sequence was used to design primers, and amplicons of approximately 500 bp were generated, with overlapping sequences. The amplified fragments were sequenced using an ABI 3700 DNA sequencer. Our sequencing panel ($n = 16$) has over 80% power to detect SNPs with minor allele frequency over 5% (37). We also sequenced cDNA sequences from the postmortem samples reported earlier (6), but did not detect any exonic variation.

Sequence analysis and database screening. The sequences were aligned using Sequencher (version 4.5), and polymorphisms were numbered consecutively. Additional SNPs localized to NT_022030 were obtained from the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP). We also obtained genotype data from a prior study of the NIMH sample (http://zork.wustl.edu/nimh).

Polymorphism analysis. Polymorphisms were detected only in the intronic and flanking sequences of RGS4 (Fig. 1). From the resequenced panel of 16 individuals, we identified six sets of SNPs. Members of each set were in complete linkage disequilibrium in this panel (total 26 SNPs). One or more SNPs from each set were selected for TDT analysis. SNPs were further evaluated for informativeness (minor allele frequency greater than 0.1) and availability of reliable genotyping assays. The same criteria were applied to SNPs flanking RGS4 selected from the NCBI database. Tests for Hardy–Weinberg equilibrium (HWE) expectations were conducted separately for each SNP among cases, fathers, mothers and controls from each sample. No significant deviations were observed, apart from SNP1 among Indian mothers ($P < 0.0002$, uncorrected for multiple comparisons). For the analysis of IBD sharing among affected sib-pairs from the NIMH samples, we also used genotypes for markers D1S1595, D1S484, D1S1677, D1S431 and D1S1589 (16).

PCR-based assays included primers (5 pmol) with 200 μM dNTP, 1.5 mM $MgCl_2$, 0.5 U AmpliTaq Polymerase (PE Biosystems), 1 × buffer and 60 ng of genomic DNA in 10 or 20 μl reactions. The PCR conditions were 95°C for 10 min followed by 35 cycles (94°C for 45 s, 60°C for 45 s and 72°C for 1 min) and a final extension at 72°C for 7 min. The amplified products were digested with restriction endonucleases, electrophoresed on agarose gels and visualized using ethidium stain. SNPs 4 and 18 were identified as single-strand conformational polymorphisms (SSCP) (38). The NIMH samples and the adult controls were genotyped at SNP18 using a fluorescence polarization (FP)-based assay (39). Perfect correspondence was obtained between the SSCP and FP assays in 100 samples. All genotypes were read independently by two investigators.

SNPs 1, 4, 7 and 18 were assayed among the Indian samples at New Delhi (P.S./B.K.T.). Assays were identical to the Pittsburgh analyses, and SNP18 was assayed using SSCP analysis. Three samples for which the genotypes were confirmed by resequencing were included in all the assays. Seven samples genotyped at Pittsburgh were separately assayed blind to genotype at New Delhi. One discordant result was obtained.

Statistical analysis. PEDCHECK software was used to check for Mendelian inconsistencies (40). χ^2 tests were employed for comparisons between cases and unrelated controls. We also used SNPEM software based on the EM algorithm to estimate and compare haplotype frequencies (18). We utilized GENEHUNTER software for TDT analysis of individual SNPs and haplotypes, as well as analysis of IBD among affected sib-pairs (11). We also used TRANSMIT for global tests of association involving multiple haplotypes (12,13). Linkage disequilibrium was estimated using published software (41).

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REFERENCES

1. Gottesman, I. (1991) Schizophrenia Genesis: The Origins of Madness. W.H. Freeman, New York.
2. Baron, M. (2001) Genetics of schizophrenia in the new millennium: progress and pitfalls. Am. J. Hum. Genet., 68, 299–312.
3. Risch, N.J. (2000) Searching for genetic determinants in the new millennium. Nature, 405, 847–856.
4. Spielman, R.S., McGinnis, R.E. and Ewens, W.J. (1994) The transmission/disequilibrium test detects cosegregation and linkage. Am. J. Hum. Genet., 54, 559–560.
5. Mirnics, K., Middleton, F.A., Lewis, D.A. and Levitt, P. (2001) Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. Trends Neurosci., 24, 479–486.
6. Mirnics, K., Middleton, F.A., Stanwood, G.D., Lewis, D.A. and Levitt, P. (2001) Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. Mol. Psychiatry, 6, 293–301.
7. De Vries, L., Zheng, B., Fischer, T., Elenko, E. and Farquhar, M.G. (2000) The regulator of G protein signaling family. Annu. Rev. Pharmacol. Toxicol., 40, 235–271.
8. Ni, Y.G., Gold, S.J., Iredale, P.A., Terwilliger, R.Z., Duman, R.S. and Nestler, E.J. (1999) Region-specific regulation of RGS4 (regulator of G-protein-signaling protein type 4) in brain by stress and glucocorticoids: in vivo and in vitro studies. J. Neurosci., 19, 3674–3680.
9. Brzustowicz, L.M., Hodgkinson, K.A., Chow, E.W., Honer, W.G. and Bassett, A.S. (2000) Location of a major susceptibility locus for familial schizophrenia on chromosome 1q21–q22. Science, 288, 678–682.
10. Cloninger, C.R., Kaufmann, C.A., Faraone, S.V., Malaspina, D., Svrakic, D.M., Harkavy-Friedman, J., Suarez, B.K., Matise, T.C., Shore, D., Lee, H. et al. (1998) Genome-wide search for schizophrenia susceptibility loci: the NIMH Genetics Initiative and Millennium Consortium. Am. J. Med. Genet., 81, 275–281.
11. Kruglyak, L., Daly, M.J., Reeve-Daly, M.P. and Lander, E.S. (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. Am. J. Hum. Genet., 58, 1347–1363.
12. Clayton, D. and Jones, H. (1999) Transmission/disequilibrium tests for extended marker haplotypes. Am. J. Hum. Genet., 65, 1161–1169.
13. Clayton, D. (1999) A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. Am. J. Hum. Genet., 65, 1170–1177.
14. Merriman, T.R., Eaves, I.A., Twells, R.C., Merriman, M.E., Danoy, P.A., Muxworthy, C.E., Hunter, K.M., Cox, R.D., Cucca, F., McKinney, P.A. et al. (1998) Transmission of haplotypes of microsatellite markers rather than single marker alleles in the mapping of a putative type 1 diabetes susceptibility gene (IDDM6). Hum. Mol. Genet., 7, 517–524.
15. Nair, R.P., Stuart, P., Henseler, T., Jenish, S., Chia, N.V., Westphal, E., Schork, N.J., Kim, J., Lim, H.W., Christophers, E. et al. (2000) Localization of psoriasis-susceptibility locus PSORS1 to a 60-kb interval telomeric to HLA-C. Am. J. Hum. Genet., 66, 1833–1844.
16. Faraone, S.V., Matise, T., Svrakic, D., Pepple, J., Malaspina, D., Suarez, B., Hampe, C., Zambuto, C.T., Schmitt, K., Meyer, J. et al. (1998) Genome scan of European–American schizophrenia pedigrees: results of the NIMH Genetics Initiative and Millennium Consortium. Am. J. Med. Genet., 81, 290–295.
17. Fallin, D. and Schork, N.J. (2000) Accuracy of haplotype frequency estimation for biallelic loci, via the expectation-maximization algorithm for unphased diploid genotype data. Am. J. Hum. Genet., 67, 947–959.
18. Fallin, D., Cohen, A., Essioux, L., Chumakov, I., Blumenfeld, M., Cohen, D. and Schork, N.J. (2001) Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. Genome Res., 11, 143–151.
19. Lewontin, R.C. (1988) On measures of gametic disequilibrium. Genetics, 120, 849–852.
20. Seltman, H., Roeder, K. and Devlin, B. (2001) Transmission/disequilibrium test meets measured haplotype analysis: family-based association analysis guided by evolution of haplotypes. Am. J. Hum. Genet., 68, 1250–1263.
21. Reich, D.E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P.C., Richter, D.J., Lavery, T., Kouyoumjian, R., Farhadian, S.F., Ward, R. and Lander, E.S. (2001) Linkage disequilibrium in the human genome. Nature, 411, 199–204.
22. Lyman, R.F., Lai, C. and MacKay, T.F. (1999) Linkage disequilibrium mapping of molecular polymorphisms at the scabrous locus associated with naturally occurring variation in bristle number in Drosophila melanogaster. Genet. Res., 74, 303–311.
23. Horikawa, Y., Oda, N., Cox, N.J., Li, X., Orho-Melander, M., Hara, M., Hinokio, Y., Lindner, T.H., Mashima, H., Schwarz, P.E. et al. (2000) Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat. Genet., 26, 163–175.
24. Ober, C., Leavitt, S.A., Tsalenko, A., Howard, T.D., Hoki, D.M., Daniel, R., Newman, D.L., Wu, X., Parry, R., Lester, L.A. et al. (2000) Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations. Am. J. Hum. Genet., 66, 517–526.
25. Ewens, W.J. and Spielman, R.S. (1995) The transmission/disequilibrium test: history, subdivision, and admixture. Am. J. Hum. Genet., 57, 455–464.
26. Spielman, R.S. and Ewens, W.J. (1996) The TDT and other family-based tests for linkage disequilibrium and association [editorial]. Am. J. Hum. Genet., 59, 983–989.
27. Bacanu, S.A., Devlin, B. and Roeder, K. (2000) The power of genomic control. Am. J. Hum. Genet., 66, 1933–1944.
28. Wildenauer, D.B., Schwab, S.G., Maier, W. and Detera-Wadleigh, S.D. (1999) Do schizophrenia and affective disorder share susceptibility genes? Schizophr. Res., 39, 107–111; discussion 160.
29. Berrettini, W.H. (2000) Are schizophrenic and bipolar disorders related? A review of family and molecular studies. Biol. Psychiatry, 48, 531–538.
30. Sun, F., Flanders, W.D., Yang, Q. and Khoury, M.J. (1999) Transmission disequilibrium test (TDT) when only one parent is available: the 1-TDT. Am. J. Epidemiol., 150, 97–104.
31. Brown, G.W. and Birley, J.L.T. (1969) Crises and life changes and the onset of schizophrenia. J. Health. Soc. Behav., 9, 203–214.
32. Agid, O., Shapira, B., Zislin, J., Ritsner, M., Hanin, B., Murad, H., Troudart, T., Bloch, M., Heresco-Levy, U. and Lerer, B. (1999) Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. Mol. Psychiatry, 4, 163–172.
33. Arango, C., Kirkpatrick, B. and Koenig, J. (2001) At issue: stress, hippocampal neuronal turnover, and neuropsychiatric disorders. Schizophr. Bull., 27, 477–480.
34. Nurnberger, J.I.Jr., Blehar, M.C., Kaufmann, C.A., York-Cooler, C., Simpson, S.G., Harkavy-Friedman, J., Severe, J.B., Malaspina, D. and Reich, T. (1994) Diagnostic interview for genetic studies. Rationale, unique features, and training. NIMH Genetics Initiative. Arch. Gen. Psychiatry, 51, 849–859; discussion 863–864.
35. Deshpande, S.N., Mathur, M.N.L., Das, S.K., Bhatia, T., Sharma, S.D. and Nimgaonkar, V.L. (1998) A Hindi version of the Diagnostic Interview for Genetic Studies. Schizophr. Bull., 24, 489–493.
36. Druey, K.M., Blumer, K.J., Kang, V.H. and Kehrl, J.H. (1996) Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. Nature, 379, 742–746.
37. Kruglyak, L. and Nickerson, D.A. (2001) Variation is the spice of life. Nat. Genet., 27, 234–236.
38. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl Acad. Sci. USA, 86, 2766–2770.
39. Chen, X. and Kwok, P.Y. (1997) Template-directed dye-terminator incorporation (TDI) assay: a homogeneous DNA diagnostic method based on fluorescence resonance energy transfer. Nucleic Acids Res., 25, 347–353.
40. O'Connell, J.R. and Weeks, D.E. (1998) PedCheck: a program for identification of genotype incompatibilities in linkage analysis. Am. J. Hum. Genet., 63, 259–266.
41. Zhao, J.H., Curtis, D. and Sham, P.C. (2000) Model-free analysis and permutation tests for allelic associations. Hum. Hered., 50, 133–139.