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ORIGINAL ARTICLE

Panic disorder is associated with the serotonin transporter gene (*SLC6A4*) but not the promoter region (5-HTTLPR)

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Panic disorder (PD) and social anxiety disorder (SAD) are moderately heritable anxiety disorders. We analyzed five genes, derived from pharmacological or translational mouse models, in a new case–control study of PD and SAD in European Americans: (1) the serotonin transporter (SLC6A4), (2) the serotonin receptor 1A, (3) catechol-*O*-methyltransferase, (4) a regulator of g-protein signaling and (5) the gastrin-releasing peptide receptor. Cases were interviewed using the schedule for affective disorders and schizophrenia and were required to have a probable or definite lifetime diagnosis of PD ($N=179$), SAD (161) or both (140), with first onset by age 31 and a family history of anxiety. Final diagnoses were determined using the best estimate procedure, blind to genotyping data. Controls were obtained from the National Institute of Mental Health Human Genetics Initiative; only subjects above 25 years of age who screened negative for all psychiatric symptoms were included ($N=470$). A total of 45 single nucleotide polymorphisms were successfully genotyped over the five selected genes using Applied Biosystems SNPlex protocol. SLC6A4 provided strong and consistent evidence of association with the PD and PD+SAD groups, with the most significant association in both groups being at rs140701 ($\chi^2=10.72$, $P=0.001$ with PD and $\chi^2=8.59$, $P=0.003$ in the PD+SAD group). This association remained significant after multiple test correction. Those carrying at least one copy of the haplotype A-A-G constructed from rs3794808, rs140701 and rs4583306 have 1.7 times the odds of PD than those without the haplotype (95% confidence interval: 1.2–2.3). The SAD only group did not provide evidence of association, suggesting a PD-driven association. The findings remained after adjustment for age and sex, and there was no evidence that the association was due to population stratification. The promoter region of the gene, *5-HTTLPR*, did not provide any evidence of association, regardless of whether analyzed as a triallelic or biallelic locus, nor did any of the other four candidate genes tested. Our findings suggest that the serotonin transporter gene may play a role in PD; however, the findings require replication. Future studies should attend to the entire genetic region rather than the promoter.

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Introduction

We report on a candidate gene study for panic disorder (PD) and social anxiety disorder (SAD). Anxiety disorders as a group are the most prevalent psychiatric disorders in the community. Although they are classified as discrete disorders, comorbidity among them is high and they often respond to the same treatments. Anxiety disorders are of interest to

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study genetically because they are moderately heritable; furthermore, the neural circuitry required for learned and innate fear is well worked out in both human and animal models.[1–3] This neural circuitry can suggest candidate genes that can be tested with disease related variants in clinical samples.

There are no clear guides as to which of the anxiety disorders are more likely than others to share common genetic etiology. We elected to study PD and SAD because they can be clinically well defined and are moderately heritable. PD is characterized by discrete and recurrent episodes of rapid sudden onset of uncontrollable fear and accompanying cardiac, respiratory and other symptoms. At least some of the symptoms appear to arrive 'out of the blue' and are not related to consistent cues. The lifetime prevalence ranges from 2 to 5%, age at first onset is in the early 20s, PD is more common in women, and there is consistency in clinical presentation cross nationally.[4,5] Family studies indicate that PD is highly familial (for example,[6–9]) with the risk to first-degree relatives of PD probands with onset under the age of 20 years being 17-fold, and under 30 years, sixfold, as compared with risk in relatives of controls.[6,7] A meta-analysis of twin and family studies suggests a heritability of about 48%.[8]

Phobias are also a clinically heterogeneous group of conditions with a common feature of avoidance behavior secondary to irrational fears of a specific activity, object or situation. The most consistent data suggesting genetic heritability among the phobias concern social phobias, termed interchangeably as SAD, in the Diagnostic and Statistical Manual of Mental Disorders (DSM IV). SAD includes a concern about appearing shameful or stupid in the presence of others, resulting in a persistent fear of public performance and of situations in which there is possible eating, bathing in public or use of public lavatories. Exposure to a phobic stimulus pushes an immediate anxiety response, and the phobic situation is avoided or is endured with intense anxiety. The lifetime prevalence varies with ranges for generalized social phobia of 3–4% in the United States, mean age-of-onset in the early teens and higher prevalence in women than men.[1–3] Although family studies for SAD are fewer than for PD, they indicate over threefold increase of SAD, especially generalized SAD, in first-degree relatives of probands with SAD.[4–6] Heritability of SAD based on twin studies is similar to that for PD.[1] Although other anxiety disorders likely have a genetic component in their etiology, their evidence is limited or more equivocal.[7,8]

Linkage and association studies have identified genes potentially related to the etiology of PD[9–11] and SAD.[6,12,13] We had a large and well-defined sample of PD and SAD cases, and we used this sample to follow-up on genes that have been reported in the literature to be associated with PD or SAD phenotypes. We choose five genes, derived from pharmacological or translational mouse models, to analyze in a case–control association study: (1) the serotonin transporter, SLC6A4, (2) the serotonin receptor 1A (HTR1A), (3) catechol-*O*-methyltransferase (COMT), (4) a regulator of g-protein signaling (RGS2) and (5) the gastrin-releasing peptide receptor (GRPR). We choose 45 single nucleotide polymorphisms (SNPs) for typing in these five genes, as shown in Table 1.

Substantial evidence points to the involvement of the serotonin system in PD and SAD, notably the effectiveness of drugs such as monoamine oxidase inhibitors and selective-serotonin reuptake inhibitors (SSRIs) in treatment.[14] SLC6A4, which is the primary target of SSRIs, and HTR1A have been studied extensively in these disorders, with reports of both positive and negative findings.[15] However, the majority of these studies assayed only one or two select polymorphisms in case–control studies of relatively small sample size. Most of these studies focused on the functional serotonin transporter promoter polymorphism, 5-HTTLPR, again with conflicting results,[15] although a recent meta-analysis concludes that there is no relationship with PD.[16] Although these discrepancies in the promoter may be attributable to genetic heterogeneity, phenotype definition or variable power, it is also possible that the association is with another region of the gene, or that the standard subdivision of this allele into long (L) and short (S) may be oversimplified. A recent report identified 5-HTTLPR as functionally triallelic, with a common A/G substitution within the L allele creating a functional AP2 transcription-factor binding site and resulting in reduced gene expression comparable to that of the S allele.[17] We have incorporated this finding in our evaluation of 5-HTTLPR.

The catechol-*O*-methyltransferase gene, which codes for one of the major methylation enzymes metabolizing monoaminergic neurotransmitters including dopamine, has repeatedly been suggested as a promising candidate gene in the pathogenesis of PD.[18–20] The COMT gene maps to chromosome 22q11.2 where it is expressed as a larger, membrane-bound protein or a smaller, soluble form. Both forms encode a functional polymorphism (val158met) wherein individuals harboring the valine allele show significantly higher COMT activity relative to those harboring the methionine allele.[21,22] A number of independent case–control and/or family-based association studies have been reported comparing the functional COMT val158met polymorphism with anxiety disorder; some showing no evidence of association[23,24] whereas others report association with the valine allele,[25–27] or conversely the methionine allele.[28,29] A recent meta-analysis of six studies with total sample size comparable to the present study reported significant association of the COMT 158val allele with PD in Caucasian samples.[30] In a recent study of prefrontal working memory response, it was reported that the val158met variant exerts its phenotypic effect by interaction with two additional COMT variants located just upstream and downstream of the gene-coding region.[31]

Table 1 The 45 SNPs analyzed in the five candidate genes

db SNP Build 128				
Gene	*5′*	*3′*	*Chromosomal location (bp)*	*SNP*
RGS2	191 044 794	191 048 026	191 044 782	rs12130714
			191 045 850	rs2746073
			191 047 121	rs17647363
			191 047 795	rs4606
			191 048 262	rs3767488
5HTR1A	63 292 034	63 293 302	63 292 485	rs1800042
			63 292 752	rs1800043
			63 293 009	rs6294
			63 293 256	rs1800041
			63 294 321	rs6295
SLC6A4	25 549 032	25 586 831	25 549 137	rs1042173
			25 554 071	rs2054848
			25 555 919	rs3794808
			25 562 658	rs140701
			25 562 841	rs4583306
			25 566 514	rs717742
			25 567 515	rs140700
			25 571 040	rs2020942
			25 572 936	rs6355
			25 574 940	rs2020936
			25 575 791	rs2066713
			25 583 308	rs8073965
			25 585 881	rs2020933
			25 588 134	5-HTTLPR_HAPTYPE
MB-COMT	18 309 309	18 336 530	18 308 092	rs2097603[a]
S-COMT	18 330 070	18 336 528	18 310 109	rs737866
			18 311 407	rs933271
			18 311 668	rs1544325
			18 314 051	rs174675
			18 317 638	rs5993883
			18 321 947	rs5992500
			18 325 177	rs740603
			18 330 235	rs4633
			18 330 268	rs740602
			18 331 201	rs769223
			18 331 271	rs4680
			18 332 132	rs4646316
			18 333 176	rs174696
			18 336 781	rs165599
GRPR	16 051 345	16 081 562	16 049 496	rs12850070
			16 057 042	rs10218163
			16 064 270	rs7876221
			16 080 998	rs3747411
			16 081 881	rs2353576
			16 084 478	rs6527664

Abbreviations: GRPR, gastrin-releasing peptide receptor; MB-COMT, membrane-bound catechol-*O*-methyltransferase; RGS2, regulator of g-protein signaling; S-COMT, soluble form of catechol-*O*-methyltransferase; SNP, single nucleotide polymorphism.

Information on bp position and the extent of the coding region 5′–3′.

[a] rs2097603 is the same as rs2075507. The two SNPs that reportedly interact with rs4684 to affect a working memory phenotype (rs2097603 and rs165599; Meyer-Lindenberg *et al.*[31]) map approximately 2000 bp proximal to rs737866 and 3600 bp distal to rs174696, respectively.

The final two genes, RGS2 and GRPR, have been shown to be involved in mouse models of anxiety and fear responses, respectively.[32–34] Furthermore, significant evidence for association to variants in RGS2 has been reported in a sample of 173 patients with PD and 173 controls.[35]

Our goal was to determine whether SNPs in any of the five candidate genes were associated with any or all of the three diagnostic categories in our sample: PD, SAD or the combined diagnosis of PD and SAD, and to determine whether that phenotype could be further refined by comorbidities or age-of-onset. Comorbid conditions, such as agoraphobia and specific phobia, are common among these PD and SAD diagnostic categories. In addition, there is evidence that PD with onset before age 20 may represent a more genetically heritable form of the disease,[36,37] with pre-pubescent patients alone (onset before 12 years of age) studied only minimally. We thus further assessed (1) whether any association between the PD and SAD diagnostic groups and the candidate genes could be refined using the available sample information on the comorbid conditions of agoraphobia, specific phobia or early-onset recurrent depression (recurrent major depressive disorder, MDD); and (2) whether there was an age-of-onset component to the association evidence; that is, whether the association was stronger in those with an earlier age-of-onset (<20 or 12) than in those with a later onset.

Materials and methods

Subjects

Sample recruitment and characteristics have been previously detailed.[38] Briefly, PD and SAD subjects in the age range of 18–65 were recruited between May 2004 and December 2006, with New York State Psychiatric Institute's Institutional Review Board approval. Requirements for inclusion included: a probable or definite diagnosis of PD with or without agoraphobia, SAD or both; a family history of anxiety in at least one first-degree relative; and first onset of the full syndrome prior to age 31. Agoraphobia was not required, as our family study data showed no increase in familial aggregation of panic by whether or not the proband with PD had agoraphobia.[39] Patients with SAD were required to have the generalized form, including at least three situations that elicit extreme social anxiety.[6] Subjects with a history of bipolar disorder, schizophrenia or antisocial personality; those for whom a medical or neurological disorder or treatment might explain the anxiety symptoms; and those who had participated in any of our other genetic studies were excluded prior to DNA collection. For the primary analyses we included only European American cases and National Institute of Mental Health (NIMH) controls. A sample of African American cases and controls were also analyzed in an exploratory manner for association at SLC6A4.

Psychiatric diagnoses were ascertained using the Schedule for Affective Disorders and Schizophrenia-Lifetime Version modified for the study of anxiety disorders and updated for DSM IV (SADS-LA IV).[40] Information on first-degree relatives was obtained using the Family History Screen, with the proband as the informant.[41] All diagnostic assessments were administered by trained interviewers who were doctoral- and master's-level mental health professionals. Interviewers were required to complete 3–4 page narrative summaries following a common format. The narrative included historical summary of all symptoms, descriptions of mental status and explanation for all uncertain ratings. In addition, basic demographic and medical history was obtained. Final psychiatric diagnoses were made by MMW blinded to subjects' genetic data, using the best estimate procedure.[42] Any cases of diagnostic uncertainty were reviewed by AJF and were reinterviewed or eliminated if the diagnosis could not be made with certainty.

DNA for control subjects was obtained from the NIMH Human Genetic Initiative (http://www.nimhgenetics.org). A total of 2959 samples were provided to us at the time of analyses. All subjects were European American or African American. Of these, we excluded subjects who displayed any symptoms of schizophrenia, schizoaffective disorder, bipolar disorder, any anxiety or depressive disorder, or substance abuse or dependence, resulting in a sample of 838 (28%) subjects. We then further excluded women under age 25 and men under age 30 to minimize the likelihood that we were including subjects who were still at risk for developing PD. The final sample consisted of 470 European Americans and 61 African Americans, 282 were women and 269 were men (with the lower cutoff for women selected to better match controls to the predominantly female cases).

Psychiatric symptoms in the controls were assessed at the time of their original recruitment by the NIMH by an online self-report that included demographic and medical information, and a psychiatric history based on the Composite International Diagnostic Interview-Short Form[43] (see Talati *et al.*[44] for detailed description). We did not assess the control subjects in the present study.

Lab methods

A total of 45 SNPs were successfully genotyped over the 5 selected candidate genes using Applied Biosystem's SNPlex protocol.[45] SNPs were selected for genotyping across each gene based on the following procedure: (1) linkage disequilibrium (LD) patterns across the gene were determined using Haploview[46] with HapMap CEPH genotype data (www.hapmap.org). Then the tagger algorithm, with an r^2 threshold of 0.8, was used to choose a set of tagSNPs over each gene. (2) The literature was searched for variants in the candidate genes that had been specifically implicated in any of the phenotypes under investigation.

5-HTTLPR genotyping was carried out in two stages, first discriminating between the L and S alleles and second determining the status of the A/G SNP within the L allele (L_A/L_G). Stage one genotyping was carried out as described in Yonan *et al.*[47] In the second stage of genotyping, the remaining PCR product of those samples with an L allele was digested with 10 U of *Hpa*II for 4 h at 37 °C, followed by 20 min at 65 °C, and products resolved on the ABI3730XL as before. The presence of the G nucleotide creates a restriction enzyme cut site, resulting in either a 155 or 329 bp product corresponding to the L_G or L_A allele, respectively.

Statistical methods

All SNP association analyses were conducted using Splus (7.0 for Windows, Insightful Corp.) and STATA (Stata/SE 9.0 for Windows, College Station, TX, USA). At each of the 45 SNPs a trend test was used to assess the association evidence between markers in the candidate genes and the diagnostic groups and subtypes, adjusted for gender and age using logistic regression. The 5-HTTLPR triallelic marker in SLC6A4 was assessed for association as both a trialleic and biallelic variant, and independently based upon the A/G SNP variant within the 5-HTTLPR L allele. We corrected for multiple hypothesis tests by using a Bonferroni correction, as it provides the most conservative adjustment and our SNPs were chosen using a tagging approach. D' was calculated to measure the degree of LD in our control sample. Haplotypes were constructed using phase 2.1.1.[48] To estimate the haplotypes, we used 5000 iterations, 1 thinning interval and 5000 burn-ins. The positions of the markers were not specified. Multiple runs varying the seed were used to determine whether the phase assignments were consistent. We tested for differences in haplotype and haplogenotype frequencies between cases and controls using χ^2-statistics. We also computed odds ratios (ORs) with 95% confidence intervals (CIs).

An additional 90 neutral SNPs were genotyped in the sample to test for the existence of population stratification. We selected SNPs from the http://rosenberglab.bioinformatics.med.umich.edu/datasets.html website.[49,50] The site represents the allele frequencies for 8700 SNPs derived from genotypic analysis of 42 East-Asian, 42 African-American and 42 European-American samples. For each SNP, the site then lists four 'I_n statistic' values that measure ability to infer ancestry from each population comparison, and thereby assesses the utility of the marker for detecting population stratification. Employing the most general statistic (I125), the average I_n for the 90 markers is 0.23 and the minimum value is 0.19. Only 1.5% of the 8700 SNPs characterized in Rosenberg *et al.*[49] were more informative. These 90 SNPs are provided in Table 4.

We used the program STRUCTURE[51] and the DC method[52,53] to test for the existence of population stratification. We used STRUCTURE to determine whether our 90 SNPs could detect any substructure in our combined sample of cases and controls of reported European-American ancestry. We then used the DC method to assess whether any substructure in the data differed between cases and controls and could result in population stratification. Using this DC approach, one can estimate the amount of population stratification in one's sample and test whether this estimate differs significantly from zero. If so a correction factor is employed in the same way (although the correction factor differs) as one would use the genomic control λ.[54]

Results

Sample characteristics

The analyzed sample comprised 409 cases (PD, $N=163$; SAD, 130; PD + SAD, 119) and 470 controls. Table 2 shows the distribution of cases and controls by age and gender. All study participants, unless noted otherwise, were European American (note that the controls were chosen to be older, so as to minimize their likelihood of still being at risk for PD or SAD). Comorbid conditions were common among the PD and SAD cases, as shown in Table 2. For

Table 2 Demographic characteristics and comorbidity among European-American panic and/or social anxiety disorder cases, and controls

	Panic (N = *163*)	*Social anxiety* (N = *130*)	*Panic + social* (N = *119*)	*Controls* (N = *470*)
Demographics				
% Female	81	77	80	49
Median age (range), years	35 (19–66)	35 (19–64)	36 (18–64)	56 (26–75)
Comorbid conditions				
Recurrent MDD[a]	53 (33)	34 (26)	54 (45)	NA
Specific phobia	56 (34)	33 (25)	50 (42)	NA
Agoraphobia	130 (80)	3 (2)	80 (67)	NA
Age-o-onset				
Panic onset ⩽12 years	29 (16)	NA	25 (21)	NA
Panic onset ⩽20 year	79 (44)	NA	72 (61)	NA

Abbreviation: MDD, major depressive disorder; NA, not applicable.
[a] Early-onset recurrent major depressive disorder, with first onset by age 30.

example, 142 (31%) of all cases also had early-onset recurrent MDD (defined as age-of-onset <30 years), and 139 (34%) had specific phobia. Among the panic cases, 19.5% had had their first onset by age 12, and over half (52%) by age 20. A small sample of African-American cases with PD or PD + SAD ($N = 24$) and NIMH controls ($N = 81$) were analyzed in an exploratory manner for association with SLC6A4.

PD and SAD single SNP association study

Of all the 45 SNPs tested in the 5 candidate genes, only SNPs in SLC6A4 provided evidence of association with PD and PD + SAD, at the 5% level. This gene continued to provide significant evidence of association, even after Bonferroni correction for the 45 tests of association, and after adjustment for age and sex. The estimated ORs remained similar after age and sex adjustment. The promoter region of the gene, *5-HTTLPR*, did not provide significant evidence of association with PD or PD + SAD, regardless of whether it was analyzed as triallelic, biallelic or a straight analysis of the A/G SNP. There was little evidence of association between the SNPs in SLC6A4 and SAD alone, which indicates that the association is restricted to PD or PD + SAD; that is, it appears to be a PD association. The association evidence in this gene with PD and with the PD + SAD diagnostic group was strong and consistent. rs140701 provided the largest χ^2_1-statistics for both diagnostic groups, with $\chi^2_1 = 10.72$ ($P = 0.001$) and $\chi^2_1 = 8.59$ ($P = 0.003$) for PD and PD + SAD, respectively, and both diagnostic groups followed a similar pattern of association across the gene. One SNP near 5-HTTLPR provided some significant association evidence with SAD (rs8073965); however, the flanking SNPs provided little corroborating evidence, the minor allele frequency at this SNP is low (see Table 3), and there is a great deal of LD between this SNP and rs140701 as measured by D'. Figure 1 plots the χ^2-statistics (1 d.f. test of trend) for SNPs typed in this gene against the SNP location in kb on the x axis. The solid black line represents the association at each SNP with PD, whereas the dotted line represents the association with the phenotype of PD + SAD. The horizontal line at $\chi^2_{\mathrm{CRIT}} = 10.63$ represents the required critical value for significance at the 5% level, after taking the 45 SNP tests into account using a Bonferroni approach. The similar shapes of the PD and PD + SAD lines provide support for combining the two groups into one, and interpreting the association as one with PD, regardless of SAD status as a comorbid condition; in this case, $\chi^2_1 = 15.81$ ($P = 0.0001$) at rs140701. Table 3 lists the minor allele frequencies in the control sample.

Population stratification

To determine whether this association could be due to population stratification, we typed 90 neutral loci, looked for population substructure using STRUCTURE,[51] and then tested, using the DC method,[52,53] whether any observed structure could result in population stratification providing a spurious association result with SLC6A4.

Table 3 Minor allele frequencies (European-American controls) for SNPs in SLC6A4

SNPs in SLC6A4	*Alleles (major/minor)*	*Minor allele frequency in controls*
rs1042173	G/T	0.436
rs2054848	G/A	0.003
rs3794808	A/G	0.427
rs140701	A/G	0.408
rs4583306	G/A	0.408
rs717742	T/A	0.203
rs140700	A/G	0.091
rs2020942	A/G	0.380
rs6355	C/G	0.030
rs2020936	C/T	0.197
rs2066713	T/C	0.383
rs8073965	T/G	0.025
rs2020933	A/T	0.073
5-HTTLPR_HAPTYPE	NA	NA

Abbreviation: SNP, single nucleotide polymorphism; NA, not applicable.

In STRUCTURE we used 300 000 burn-ins and 300 000 replications with the 90 neutral SNPs (Table 4) for four runs each of $k = 2, 3, 4, 5, 6$ on our sample of PD and PD + SAD cases and controls. At $k = 3$ there appeared to be a plateau in the likelihood providing evidence of three clusters in our sample, with relatively consistent information across the four runs at $k = 3$. One of the runs provided evidence of as many as 86% of the sample loading greater than 80% in one of the three clusters (see Figure 2). Thus, the 90 neutral SNPs appear to be able to detect structure in the combined sample of European-American cases and controls. Yet, Figure 2 also illustrates that the cases and controls are well distributed across the three clusters. In fact, when the average loadings across the three clusters were calculated, these averages were similar between cases and controls. This indicates that although there may be structure in the data, the cases and controls do not appear to differ in frequency across the clusters; an indication that the cases and controls are comparable with respect to ancestry. The DC method allows us to formally test whether there is a difference between the cases and controls that could result in population stratification.

In the DC method, one first tests for the existence of population stratification due to allele frequency differences between cases and controls using a t-test, which tests whether the DC statistic differs from zero. From our 90 neutral loci, we calculated $\hat{\delta} = -0.1545$; a value which can be interpreted as very small.[53] This resulted in an observed t-statistic of -0.3980 ($P = 0.694$), indicating that even though there is some structure in the combined sample of European-American cases and controls as uncovered

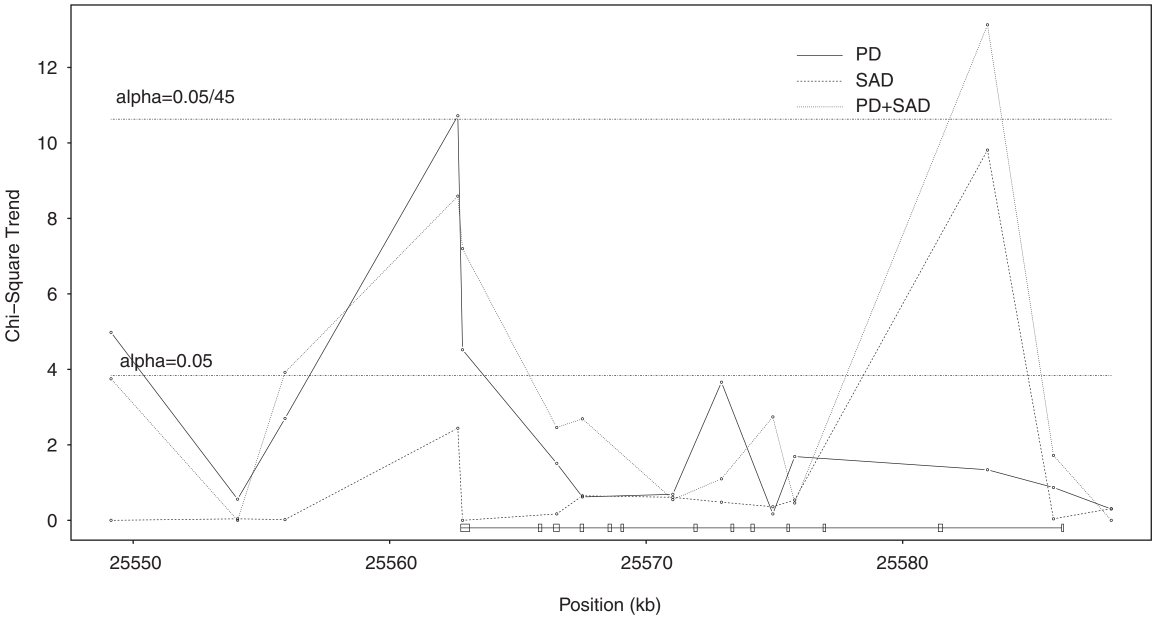

Figure 1 Association between SNPs in SLC6A4 and panic disorder (PD), social anxiety disorder (SAD) or PD + SAD, χ^2-critical value corresponding to Bonferroni adjustment for 45 SNPs studied in 5 candidate genes, is provided by the top dotted line. The location of the genotyped SNPs relative to the SLC6A4 gene is depicted just above the *x* axis.

in the STRUCTURE analysis, the cases and controls do not appear to differ from each other significantly based on the set of 90 SNPs used. Thus, there is no evidence that the observed association between PD (PD + SAD) and SLC6A4 is due to population stratification.

Association of SLC6A4 haplotypes with PD

The LD in SLC6A4 was calculated from the control sample using D'. The region containing our association peak (from 25 550 to 25 570 kb) is characterized by a large amount of LD ($D' > 0.9$). However, the region from 25 570 kb appears to have lower pair-wise LD. It is interesting to note that, although rs140701 and rs8073965 are 20 kb apart, $D' > 0.8$, *while the pair-wise LD between rs140701 and other SNPs surrounding rs8073985 is negligible.*

Haplotype analysis using phase 2.1.1, allowed us to determine whether there were haplotypes or haplogenotypes in SLC6A4 that were significant predictors of PD. Haplotype construction of SNPs across the gene indicated that a three SNP haplotype and haplogenotype constructed from rs3794808, rs140701 and rs4583306 was associated with PD (the PD phenotype included those with PD or PD + SAD). The odds of PD in those with at least one copy of the haplotype A-A-G was 1.7 times the odds of those without at least one copy of the haplotype (95% CI: 1.2–2.3, $P = 0.0017$). Whereas those who had two copies of the G-G-A haplotype were 1.8 times more likely to not have PD (95% CI: 1.2–2.5, $P = 0.0009$) than those with any other genotype. There was no evidence of a haplotype in the region of 5-HTTLPR and/or rs8073965 that differed significantly in frequency between cases and controls.

Possible confounders

To avoid confounding due to ethnicity, we removed a sample of African-American cases and controls from the primary analysis. This African-American sample was analyzed separately at SLC6A4 to assess heterogeneity. We had 24 African Americans with PD or PD + SAD, and 81 NIMH African-American controls. In this small sample, there does appear to be some evidence of association at rs140701 in the African-American sample ($\chi^2(1) = 3.22$, $P = 0.07$; OR (95% CI) = 0.535 (0.27–1.07)). This indicates that SLC6A4 might be associated with PD in both European and African Americans; however, this requires further follow-up.

It is also of interest to determine whether other comorbid disorders, such as agoraphobia, recurrent MDD and specific phobia, are associated with SLC6A4. As patients were ascertained on the basis of whether they had PD or SAD, and only PD was associated with SNPs in SLC6A4, it is possible that PD status may be confounding the relationship between SLC6A4 and an alternative phenotype. We used logistic regression in the subsample of those with PD (omitting all NIMH controls from these analyses), determining the relationship between SNPs in SLC6A4 and those with (1) specific phobia, (2) recurrent MDD and (3) agoraphobia. As before, adjusted and unadjusted (for age and sex) analyses were conducted.

The breakdown in the data indicated that those with agoraphobia had primarily PD or PD + SAD diagnoses, as expected (Table 2), whereas specific phobia and recurrent MDD were more evenly distributed across the PD and SAD diagnostic groups. There was no evidence of association between any of

Table 4 The 90 neutral SNPs typed for population stratification analysis

SNP	*Chromosome*	*Position (bp)*
rs716924	1	36 196 924
rs2038026	1	23 720 557
rs15864	1	110 094 378
rs1554615	1	198 544 703
rs1040501	1	167 354 937
rs1015140	1	198 545 381
rs729253	2	117 648 706
rs714649	2	157 849 337
rs163077	2	38 139 108
rs878172	3	71 705 138
rs592275	3	40 787 980
rs317575	3	4 063 808
rs1983273	3	190 066 320
rs1568598	3	190 012 861
rs1443529	3	64 374 558
rs1399272	3	103 294 465
rs1108718	3	65 779 622
rs1107043	3	61 813 448
rs1588041	4	117 366 454
rs1525760	4	117 354 828
rs1506739	4	117 373 198
rs1485768	4	177 877 416
rs1395433	4	117 330 553
rs1385737	4	177 901 866
rs871722	5	17 490 493
rs729800	5	133 769 779
rs434363	5	117 000 414
rs430952	5	117 005 715
rs217776	5	37 386 146
rs216377	5	37 372 467
rs2059849	5	66 646 665
rs1156387	5	103 953 546
rs1078703	5	153 545 632
rs860751	6	14 741 351
rs714389	6	83 638 065
rs276497	6	137 388 819
rs276477	6	137 383 666
rs22662	6	5 128 534
rs2180052	6	170 431 913
rs2078265	6	131 803 369
rs1455201	6	104 797 083
rs1358716	6	139 487 696
rs1322393	6	137 376 638
rs1076782	6	134 774 571
rs880028	7	50 537 629
rs739611	7	24 350 306
rs722103	8	40 618 275
rs2001433	8	10 940 884
rs2001329	8	11 024 268
rs1455640	8	2 306 616
rs998599	9	17 690 365
rs1888952	9	16 248 117
rs947603	10	95 239 594
rs1904649	10	68 626 830
rs1904648	10	68 626 960
rs757080	11	60 598 809
rs729404	11	60 977 693
rs725192	11	83 013 213
rs620778	11	120 515 009
rs612415	11	60 616 461
rs1806995	11	131 689 089
rs917587	12	3 412 935
rs1548837	12	12 945 583
rs751531	13	59 020 753
rs748144	13	112 149 884
rs1337038	13	73 624 564
rs741272	14	88 935 704
rs730570	14	100 212 642
rs716873	14	91 116 456
rs1951033	14	100 205 070
rs1872234	15	78 877 137
rs1863459	15	24 443 768
rs1426208	15	24 392 346
rs764551	16	73 761 782
rs67302	16	64 379 299
rs1019800	16	75 906 702
rs717742	17	25 566 513
rs4583306	17	25 562 840
rs3794808	17	25 555 919
rs2054848	17	25 554 070
rs140701	17	25 562 657
rs1042173	17	25 549 136
rs1833422	18	24 936 256
rs753842	19	4 928 743
rs1806931	19	15 700 364
rs733578	20	20 901 309
rs293554	20	30 549 517
rs1001519	20	58 223 865
rs1008552	21	24 599 352
rs739200	22	34 762 282

Abbreviation: SNP, single nucleotide polymorphism.

the SNPs in SLC6A4 and recurrent MDD, specific phobia or agoraphobia in the subsample of PD cases, with rs140701 resulting in *P*-values of 0.431, 0.411 and 0.074, respectively, for the three groups. Thus, none of these subtypes could further refine the PD-SLC6A4 association.

Age-of-onset analysis

The association evidence in SLC6A4 appears to be pointing to a relationship with PD, regardless of the age-of-onset of the PD (Figure 1). Yet, there is evidence that early-onset PD ($<$age 20) may represent a more genetic, or at least more familial, form of the disease.[36] As prepubertal-onset PD is uncommon, we also examined the association with PD onset <12 years of age. We thus repeated our case–control analysis restricting our sample to those with PD (in the PD or PD + SAD diagnostic groups), and treating those with onset of PD <20[12] as the cases, and those with onset greater than or equal to 20[12] as the controls. We did not find any evidence that the relationship to SNPs in SLC6A4 differed between the cases and controls, for either age cutoff ($P=0.77$ and 0.17 for ages 20 and 12, respectively, at rs140701). Thus, we were not able to further refine the PD-SLC6A4 association by age-of-onset either.

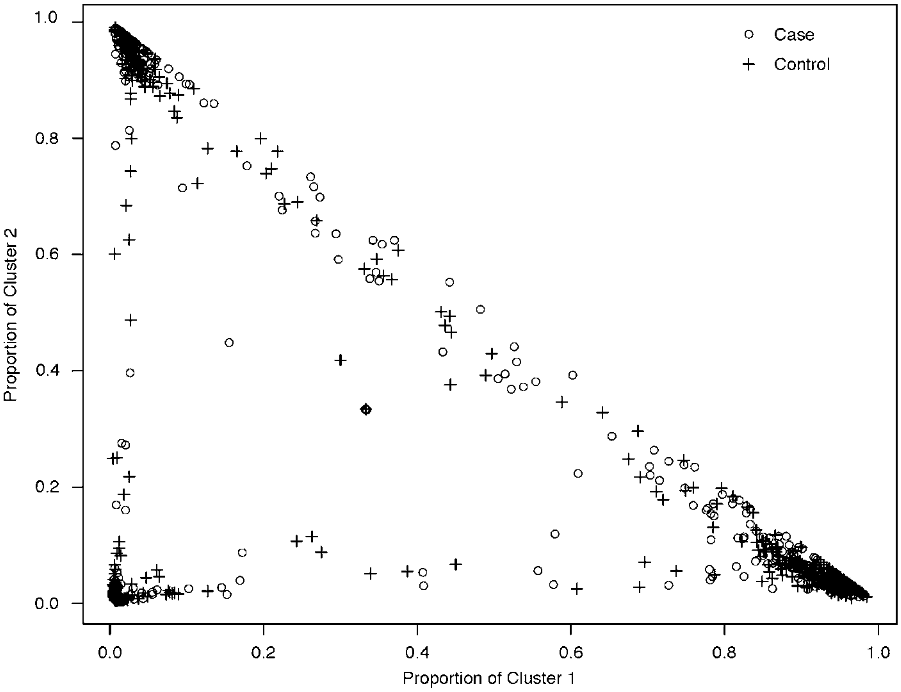

Figure 2 Clustering results from STRUCTURE, with $k = 3$ in the European-American sample of cases and controls. The proportional membership in cluster 1 is plotted against the proportional membership in cluster 2. Each '+' represents the proportional membership for a control and the circle represents the proportional membership in the three clusters for each case.

Discussion

We conducted a candidate gene association study and observed that of the five candidates we studied, only SLC6A4 appears to be significantly associated with the phenotype of PD. This PD association could not be further refined by age-of-onset or comorbidity with other psychiatric disorders, does not appear to be due to population stratification, and may also be present in African Americans, although further analysis in this group is required. In the complex system of neural communication, SLC6A4 is involved in the transport of serotonin from synaptic spaces to presynaptic neurons, thereby maintaining the pool of available serotonin for subsequent release.[55] SLC6A4 knockout mice consistently show increased anxiety-like behavior and inhibited exploratory locomotion.[56] Furthermore, significantly lower binding of SLC6A4 in the midbrain raphe, temporal lobes and hypothalamus of patients with PD has been observed and has a significant inverse correlation of binding to the severity of PD in the former two brain regions.[15]

Importantly, even though we found a significant association with the SLC6A4 gene, we did not find any significant association evidence within the promoter region (5-HTTLPR) of the gene, which is consistent with a recent meta-analysis demonstrating that the promoter region was not associated with the disorder.[16] This meta-analysis, however, considered only the biallelic locus; we here further find there is no association with the triallelic variant either. Finally, we also did not find any association with any of the other candidate genes tested.

Recent studies suggest that regulation of SLC6A4 gene expression is determined at the 5-HTTLPR promoter region in concert with regulatory effects exerted from other locations throughout the gene unit. Martin *et al.*[57] report that at least two SNPs located in intron 1 are more highly correlated with allelic expression differences in SLC6A4 than 5-HTTLPR promoter variants, and that together the upstream promoter and intronic variants account for significantly more variance in gene expression than the promoter variants alone. One of the putative regulatory variants reported by Martin *et al.*,[57] rs2020933, was evaluated in our study and did not show significant evidence of association with the anxiety phenotypes. It is possible that rs140701, located in intron 9 of SLC6A4 represents a new regulatory variant, or that it resides in LD with such a variant.

Interestingly, a number of studies have found PD to be highly familial (for example, Weissman[39]; Hettema[58]). The absolute rate varies by methods but the findings are consistent in diverse countries, with about an eightfold median relative risk of PD in the first-degree relatives of PD subjects compared to relatives of controls. When comorbidity in probands was controlled for in the above study, the aggregation in first-degree relatives of panic probands was highly specific to PD, suggesting that panic is a distinct disorder.[36] The family studies showed that the risk of PD in first-degree relatives of probands whose PD began before age 20 was increased 17-fold as compared to the risk in relatives of not ill controls,[37] and early onset may be transmitted.[59] However, these family studies also showed that onset of PD prior to age 31 conferred a sixfold increased risk of PD to relatives. On the basis of these family study findings, we selected only cases with onset age prior to 31 for the new study. Thus patients with early-onset PD might be the most promising for genetic studies. However, neither age-of-onset group appeared to further refine our PD association.

Finally, it should be noted that the NIMH controls used in this study were not directly interviewed, and information on their first-degree relatives was not available. However, a separate study by our group,[44] comparing the NIMH controls to a clinically interviewed control sample found that the subset of subjects who did not report any psychiatric symptoms—that is, those selected as controls for this analysis—were indistinguishable from the clinically interviewed controls on neuroticism and extraversion traits, and may be representative of healthy, non-ill populations. Regardless, underreporting of psychiatric symptoms among controls would reduce the association evidence; thus, using these controls results in a conservative assessment of the relationship between the candidate genes and the anxiety disorders.

Given the efficacy of SSRIs for PD, the serotonin transporter likely plays an important mechanistic role in PD. In this report we do find association evidence for the transporter, but not within the promoter region. Further studies examining the entire genetic region rather than the promoter are therefore warranted. These findings require replication, and a genome-wide association study of PD is currently underway.

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