



Two complex genotypes relevant to the kynurenine pathway and melanotropin function show association with schizophrenia and bipolar disorder

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ABSTRACT

Prior studies of mRNA expression, protein expression, and pathway metabolite levels have implicated dysregulation of the kynurenine pathway in the etiology of schizophrenia and bipolar disorder. Here we investigate whether genes involved in kynurenine pathway regulation might interact with genes that respond to kynurenine metabolites, to enhance risk for these psychiatric phenotypes. Candidate genes were selected from prior studies of genetic association, gene expression profiling and animal models. A single nucleotide polymorphism (SNP) in each of six genes, TDO2, HM74, HM74A, MCHR1, MCHR2 and MC5R, was tested for association with phenotype (475 Caucasians, 88 African Americans with schizophrenia; 97 Caucasians, 3 African Americans with bipolar disorder; 191 Caucasian, 49 African American controls). An A allele in HM74 was significantly associated with schizophrenia and with schizophrenia plus bipolar disorder combined, odds ratios (OR) of 1.48, $p = 0.011$ and 1.50, $p = 0.007$, respectively. Augmentation of disease risk was found for the complex genotype HM74[A,any] + MCHR1[T,any] + MCHR2[C,any] which conferred an OR maximal for the combined diagnostic category of schizophrenia plus bipolar disorder (1.70, $p = 0.003$), carried by 30% of the cases. TDO2[CC] + MC5R[G, any] + MCHR2[GC] conferred an OR maximal for schizophrenia alone (4.84, $p = 0.005$), carried by 8% of schizophrenia cases. The combined risk posed by these related, complex genotypes is greater than any identified single locus and may derive from co-regulation of the kynurenine pathway by interacting genes, a lack of adequate melanotropin-controlled sequestration of the kynurenine-derived pigments, or the production of melanotropin receptor ligands through kynurenine metabolism.

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1. Introduction

The heritability of schizophrenia and bipolar disorder is generally accepted to be complex in nature, a characterization resulting from the observed non-Mendelian modes of inheritance, incomplete penetrance of genetic risk, polygenic pattern of inheritance (Gottesman and Shields, 1967)

and apparent heterogeneity of genetic causation (Allen et al., 2008). Evidence for shared susceptibility loci in schizophrenia and bipolar disorder has been found in several genome-wide association studies (Berrettini, 2000). The incomplete genetic penetrance observed for both phenotypes results from modulatory genetic background effects and from environmental influences that alter the neuro-developmental course (Dean and Murray, 2005).

Here we present genetic association data concerning a model for schizophrenia and bipolar disorder that incorporates most of these features, specifically, polygenic contribution,

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increase in protein; rather, the protein for HM74 was unchanged and the protein for HM74A was decreased in schizophrenia cases as compared to controls (Miller and Dulay, 2008).

Potential modifying genes selected for study were those for a class of melanotropin receptors with relevant effects (Miller et al., 1993) in an animal model of an auditory gating endophenotype common in individuals with schizophrenia (Nagamoto et al., 1991). Within this class of receptors, three (MCHR1, MCHR2, MC5R) reside in chromosomal regions showing association with schizophrenia and/or bipolar disorder in genome-wide association studies (Schwab et al., 1998; Levinson et al., 2000; Dick et al., 2003; Lambert et al., 2005; Lin et al., 2005) and in association studies of MCHR1, specifically (Severinsen et al., 2006).

Hypothetical points of interaction between the melanotropin receptors and the kynurenine pathway are highlighted in Fig. 1. The first involves co-stimulation of the kynurenine pathway via MC5R regulation (Taylor and Namba, 2001) of interferon-gamma expression (IFN γ , Model 1 in Fig. 1).

Decreased signaling through the MC5R receptor would lead to activation of the pathway. A second point of interaction relates to the sequestration of the potentially toxic pigment molecules generated by the pathway (Model 2 in Fig. 1). The effect of peptide agonists for the MCHR1 receptor is to decrease melanosome formation and decrease the dispersion of pigment in melanosome vesicles (Baker and Ball, 1975). In this model, the capacity of pigment sequestration and dispersion would be adversely affected by a hyper-functional MCHR1 receptor. Alternatively, the pathway produces at least one key precursor for ligands of the melanotropin receptors, picolinic acid (Model 3, Fig. 1), which in its reduced form (pipecolic acid) is the core residue in the most potent peptide ligands reported for MC5R (Bednarek et al., 2007).

Thus, the six candidate genes analyzed for association with schizophrenia and bipolar disorder were: TDO2 (4q31.2), HM74A (12q24.3), HM74 (12q24.3), MCHR1 (22q13.1), MCHR2 (6q16.3) and MC5R (18p11.21). One single nucleotide polymorphism (SNP) per gene was selected, by taking

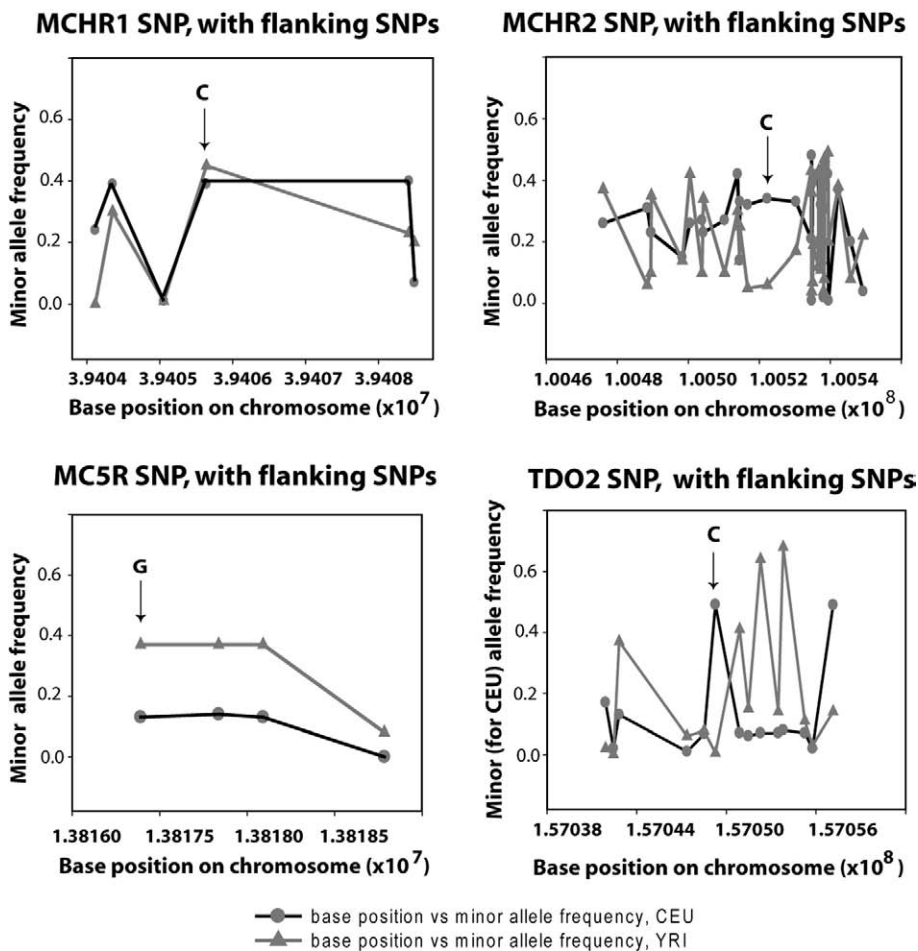


Fig. 2. Variation of SNP minor allele frequencies as reported in the Hapmap database (<http://www.hapmap.org>) for genes under study. The frequency class of allele (minor or major) was specified based on the Caucasian frequency. The minor allele for the selected SNP (marked by arrows) is noted for each gene. The circles represent minor allele frequencies for Caucasians (CEU) reported in Hapmap, and the triangles represent minor allele frequencies for Sub-Saharan Africans (YRI) reported in Hapmap. Not shown are the minor allele frequency distributions for HM74 and HM74A, since much of the data that has been collected for these genes are potentially confounded by the duplicated nature of the relevant sequences. The report for the HM74 SNP, denoted rs2454727 in the database, links to NCBI: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs2454727 which specifies a minor allele frequency (A) of 34% for the CEU population, and 24% for the YRI population.

advantage of two prior genetic association studies, one yielding positive results for HM74 (Shink et al., 2005) and the other for MCHR1 (Severinsen et al., 2006), while for untested genes, SNPs were selected that showed maximal differences in frequency between racial groups (Fig. 2). A difference between ethnic populations in disease expression and severity has been noted by many researchers studying schizophrenia (Saha et al., 2006).

2. Materials and methods

2.1. Patient population and sample acquisition

The samples as acquired were coded so as to blind the investigator to the diagnostic category and the code was broken after data submission to a 3rd party (first phase of the study) and to a co-author (SL) for the second phase of the study. The internal review boards of the University of Colorado and Johns Hopkins University approved this study. The age range of the controls at the time of evaluation was 22–96 years. The age distribution was such that approximately 1/4 of the study controls were less than 30 years of age. The sample set consisted of cases and controls of both genders, obtained from two sources: a) 323 DNA samples isolated from postmortem brain tissue obtained from the Stanley Brain Bank collection (Stanley Medical Research Institute (SMRI), Chevy Chase, MD) including 311 Caucasian individuals, 125 with schizophrenia, 97 with bipolar disorder and 89 normal controls; 12 African American individuals, 8 with schizophrenia, 3 with bipolar disorder and 1 normal control; b) 580 DNA samples isolated from immortalized lymphoblastoid cells from the University of Colorado Schizophrenia Center (Dr. Sherry Leonard), including 350 Caucasian individuals with schizophrenia, 80 African American individuals with schizophrenia, 102 Caucasian normal controls, and 48 African American normal controls. The methods for postmortem diagnosis employed by SMRI involved DSM-IV criteria employed by at least two independent psychiatrists

reviewing the records, as described by Torrey et al. (2000). The diagnostic methods employed by the University of Colorado Schizophrenia Center utilized a Structured Clinical Interview for DSM-IV Axis I Disorder (Stephens et al., 2009). A number of related individuals representing 39 Caucasian families with more than one member diagnosed with schizophrenia, composing 20% of the total schizophrenia Caucasian case population were present in the Colorado sample collection. In addition, 9 African American families with more than one member diagnosed with schizophrenia, were part of this collection, composing 21.5% of the African American case population. The analysis was therefore adjusted for the common genetic background shared by the related individuals, and differences in ethnic background and collection sites (see subsequent Statistical analyses section).

Methods employed by the SMRI Brain Bank collection are described online: (http://www.stanleyresearch.org/programs/documents/bc_statement.pdf). The SMRI collection includes adult individuals autopsied in coroner's offices located in Maine, Minneapolis, Seattle, and San Diego. The SMRI DNA was isolated from archived, frozen postmortem tissue.

The University of Colorado collection is described in more detail elsewhere (Stephens et al., 2009).

2.2. Analyses for genetic polymorphisms in candidate genes

All experimental work and determination of allele identity (Table 1) was carried by an investigator blind to the diagnostic category of each sample. The assays were carried out by TaqMan[®] PCR to distinguish alleles, using kits provided by Applied Biosystems (Foster City, CA), on the Applied Biosystems 7900HT-Fast Real-time PCR System: 95 °C 10 min, [92 °C 15 s, 60 °C 60 sec] × 47 cycles. The percent call rate was 99.86% for HM74, HM74A, and MC5R; 100% for MCHR1 and MCHR2; and 99.78% for TDO2. Samples were selected randomly for replication (approximately 4% of the sample set). The correspondence between the repeated assays and the original assays was 100%.

Table 1

SNP characteristics.

A. The database identifiers, target sequence, and related information for each SNP analyzed in this study.				
Gene	SNP rs designation	Target sequence ^a , type of polymorphism, any change in translation product, and ancestral allele.		
HM74A	N/A	CAGAGGAAGAT[G/A]ACAGGTG; nonsynonymous in sole exon; A = isoleucine instead of methionine; ancestral is likely G.		
HM74	rs2454727	CAGAGGAAGAT[G/A]ACAGGTG; nonsynonymous in sole exon; A = isoleucine instead of methionine; ancestral is likely G.		
MCHR1	rs133073	CCTCGTCTGCTGCCACTGGTCCAA[C/T]GCCAGCAACACTCTGATGGCCCG; a synonymous polymorphism in exon 1: transition substitution in exon 1; ancestral is C.		
MCHR2	rs9376618	TGCTAATGGAAACCCTGAAGACA[C/G]TTCAATAGCCACAATGTGCAAAATA; in intron 5 of full-length mRNA; transversion substitution; ancestral is G.		
MC5R	rs2236700	TGGTGTCTCTGTACATACATGTT[C/G]CTCTGGCGGACTCACGTCAAGC; nonsynonymous in sole exon; G = leucine instead of phenylalanine; ancestral is G.		
TDO2	rs2271537	TTTACTCTGCTATGCTTCTATATA[A/C]TTTTCTATTGTCAAAGAAAGAAAA; intron 4; transversion substitution; ancestral is A.		
B. Sequence of gene-specific outer primers and common inner primers (custom synthesis) used in nested PCR to distinguish the polymorphism common to both HM74A and HM74.				
Niacin-receptor duplicated gene ID	Gene-specific forward primer for outer PCR	Gene-specific reverse primer ^b for outer PCR	Common forward primer for inner PCR	Common reverse primer for inner PCR
HM74A	GCCATCATCTCTTGCTTCT	TGGCATGTTTATTTAAGGAGAGGT	TCCCAACTTCTCTCCACTTTGATC	CTCGTGCTGCGGTTAATTATCTG
HM74	GCCTAACAGTCCACCTCTG	CITCTTGAATGGTATTGTGAGGT	TCCCAACTTCTCTCCACTTTGATC	CTCGTGCTGCGGTTAATTATCTG

^a Only the target sequences, not the primer sequences for Applied Biosystems inventoried TaqMan[®] SNP products are released by the company.

^b Note that the reverse primer sequences for these genes target 4 bp of insertion/deletion that distinguishes HM74A from HM74 (Miller and Dulay, 2008).

2.3. Statistical analyses

Separate analyses were carried out comparing schizophrenic to unaffected individuals, and comparing schizophrenic plus bipolar subjects to unaffected (the number of bipolar patients was too small to be analyzed as a separate category). Various genetic models (additive, dominant, recessive, heterosis) were investigated to assess the marginal associations of six candidate SNPs to the outcome, using a logistic link function and adjusting for potential differences in ethnic groups. Generalized estimation equations (GEE; Zeger and Liang, 1986) were applied to account for relatedness of a proportion of schizophrenia cases in the University of Colorado sample collection. We also investigated potential SNP–SNP interactions in addition to the marginal SNP effects, using GEE and logic regression, an adaptive regression methodology developed specifically for this purpose (Ruczinski et al., 2003). Model selection was carried out via permutation tests and cross-validation, which addresses the multiple comparisons problem (Ruczinski et al., 2003).

All p values are reported as uncorrected p values, with the threshold for Bonferroni correction made clear. For any particular single gene model assumed (dominant, recessive, etc.), we carried out six hypothesis tests. An upper bound on the family wise error rate (FWER, i.e. the probability of at least one false rejection) can be obtained by a Bonferroni correction. Statistical significance for any of the six SNPs tested was declared if a p value of $0.05/6 = 0.0083$ or less was achieved in the final, combined data set. We did not attempt to achieve a FWER of 5% across all genetic models, since in our assessment this was not particularly meaningful (we were testing the same SNPs under different assumptions for the underlying genetic model).

The distributions of the homozygotes and heterozygotes for the 6 SNPs analyzed in this work were tested for Hardy–Weinberg Equilibrium in the controls of this sample set (Table 2).

Population stratification was previously tested and ruled out for the Colorado DNA collection (Stephens et al., 2009) by studying a series of 176 SNPs regarded as being informative for ancestry (Enoch et al., 2006). Stratification data was not available for the SMRI DNA collection.

3. Results

3.1. Association between disease and genotype at a single locus

We observed a significant association of HM74 with schizophrenia as well as schizophrenia and bipolar disease combined, after adjusting for differences in study sites and ethnic differences (Table 3). Testing a heterosis model (H), we

observed a strong association of HM74 heterozygotes with schizophrenia (OR 1.50, $p = 0.006$; Table 3), which surpassed the Bonferroni threshold for significance of $p = 0.0083$ for the 6 comparisons carried out under this particular genetic model. The locus also fit a dominant (D) genetic model (HM74[A,any]). The genotype for the HM74 dominant risk locus [A] was carried by 63% of the cases with schizophrenia, 70% of the patients with bipolar disorder and 54% of controls.

TDO2 showed a trend for association with disease, but the p value did not reach the threshold required for multiple comparisons. None of the single melanotropin loci showed a trend for risk of disease. Table 4 illustrates the data from the two sample populations (Stanley Medical Research Institute (SMRI) DNA collection and the University of Colorado DNA collection) shown separately to illustrate the significance level reached in each study.

3.2. Candidate interactions tested between kynurenine pathway and melanotropin genes

TDO2 was found to interact significantly with the melanotropin receptor polymorphisms. The interaction was suggested by the data for individuals with the TDO2[CC] genotype, where an OR 4.30, $p = 0.016$ for schizophrenia was conveyed by MC5R[G,any] plus MCHR2[GC] versus those without this melanotropin genotype (data line 3, Table 4). Across the study population, TDO2[CC] plus MC5R[G,any] plus MCHR2[GC] yielded an OR of 4.84, $p = 0.005$, when comparing schizophrenia patients to controls (data line 1, Table 4). This genotype is carried by 8% of the patients with schizophrenia, 4% of the patients with bipolar disorder and 1.6% of controls.

In addition, an interaction was present between HM74 and melanotropin receptors. In individuals with the HM74 risk genotype [A,any], the melanotropin genotypes MCHR1[T,any] plus MCHR2[C,any] conveyed an OR of 1.52 for schizophrenia, $p = 0.049$, and an OR of 1.52 for the combined diagnostic group, $p = 0.044$ (data line 4, Table 4). Across the study population, the HM74[A,any] plus MCHR1[T,any] plus MCHR2[C,any] genotype yielded an OR of 1.69, $p = 0.004$ when comparing the schizophrenia group to controls and an OR of 1.70, $p = 0.003$, for the combined diagnostic category of schizophrenia plus bipolar disorder (data line 2, Table 4). This genotype is carried by 29% of the patients with schizophrenia, 35% of the patients with bipolar disorder and 19% of controls.

A combination of both complex risk genotypes i.e., (HM74 [A,any] plus MCHR1[T,any] plus MCHR2[GC] plus MC5R[G, any] plus TDO2[CC]), was present in 16 individuals with schizophrenia, 1 individual with bipolar disorder and no controls.

Table 2

Table of Hardy–Weinberg expected and observed distributions for the controls in the sample set.

Locus	Common homozygotes expected observed	Heterozygotes expected observed	Rare homozygotes expected observed	χ^2
HM74A	102.48 110	108.04 93	28.48 36	4.63*
HM74	108.58 111	105.65 101	25.68 28	0.46
MCHR1	82.84 84	116.33 114	40.84 42	0.10
MCHR2	113.44 118	103.13 94	23.44 28	1.88
MC5R	154.89 158	74.22 68	8.89 12	1.67
TDO2	77.14 81	116.71 109	44.14 48	1.04

* $p < 0.05$.

Table 3

Odds ratios, 95% confidence intervals, and *p* values from GEE models for the associations of the six candidate SNPs to the outcome using a logistic link function and adjusting for differences in sites and race.

	Scz OR (95% CI)	<i>p</i> *	Scz + bipolar OR (95%CI)	<i>p</i> *
HM74A, ad	0.94 (0.74–1.19)	0.574	0.92 (0.73–1.15)	0.459
HM74, ad	1.23 (0.97–1.55)	0.093	1.26 (1.01–1.59)	0.044
MCHR1, ad	1.00 (0.80–1.25)	0.994	0.98 (0.80–1.21)	0.875
MCHR2, ad	1.02 (0.83–1.27)	0.823	1.00 (0.81–1.25)	0.964
MC5R, ad	1.18 (0.90–1.54)	0.232	1.11 (0.85–1.44)	0.446
TDO2, ad	1.14 (0.91–1.41)	0.252	1.16 (0.94;1.43)	0.174
HM74A, D	1.05 (0.78–1.43)	0.742	1.00 (0.75–1.35)	0.973
HM74, D	1.48 (1.10–2.00)	0.011	1.50 (1.12–2.00)	0.007
MCHR1, D	1.08 (0.79–1.49)	0.613	1.00 (0.73–1.36)	0.992
MCHR2, D	1.00 (0.73–1.36)	0.990	1.03 (0.76–1.39)	0.843
MC5R, D	1.29 (0.94–1.76)	0.113	1.19 (0.88–1.61)	0.255
TDO2, D	1.01 (0.70–1.45)	0.968	1.04 (0.73–1.49)	0.811
HM74A, R	0.69 (0.44–1.06)	0.093	0.70 (0.46–1.08)	0.110
HM74, R	0.93 (0.60–1.45)	0.749	1.04 (0.68–1.59)	0.845
MCHR1, R	0.88 (0.60–1.28)	0.488	0.95 (0.65–1.37)	0.773
MCHR2, R	1.11 (0.72–1.72)	0.631	0.95 (0.62–1.47)	0.834
MC5R, R	0.92 (0.46–1.83)	0.819	0.86 (0.43–1.69)	0.659
TDO2, R	1.40 (0.97–2.01)	0.069	1.42 (1.00–2.02)	0.050
HM74A, H	1.25 (0.93–1.69)	0.145	1.18 (0.88–1.58)	0.260
HM74, H	1.50 (1.12–1.99)	0.006	1.44 (1.09–1.90)	0.011
MCHR1, H	1.16 (0.87–1.54)	0.321	1.03 (0.78–1.36)	0.839
MCHR2, H	0.95 (0.70–1.29)	0.742	1.05 (0.78–1.42)	0.739
MC5R, H	1.33 (0.96–1.84)	0.082	1.25 (0.91–1.71)	0.168
TDO2, H	0.79 (0.58–1.07)	0.128	0.80 (0.60–1.07)	0.138

Results are shown for schizophrenia (Scz) and for the combined diagnostic category of schizophrenia and bipolar disorder (Scz + bipolar), using additive, dominant, recessive, and heterosis models (ad, D, R, and H, respectively¹).

¹HM74A, D = [A,any]; HM74, D = [A,any]; MCHR1, D = [C,any]; MCHR2, D = [C,any]; MC5R, D = [G,any]; TDO2, D = [C,any]. The recessive model (R) for each involves the homozygote of the allele specified.

*A *p* value of 0.0083 is the threshold required for Bonferonni significance, given 6 SNP comparisons.

3.3. Logic regression analysis

The logic regression method identified an interaction between diagnosis and the TDO2 and HM74 loci, adjusting simultaneously for potential differences in race and study site. The employed model selection methods addressed the multiple comparisons problem. As seen for the marginal analyses (Table 3), subjects who had two C alleles in TDO2 or at least one A allele in HM74 were at a higher risk for disease (Table 5). In addition, comparing subjects with and without genotype for TDO2 CC among those with genotype GG for HM74 (i.e. not dominant) from the same race and the same study site, the odds for the subject with TDO2[CC] are 2.93 times higher to have schizophrenia. The results for the schizophrenia plus bipolar analysis were in essence the same, except all parameter estimates achieved a higher statistical significance (Table 5).

4. Discussion

4.1. Genotypes associated with phenotype

The risk conveyed by two complex genotypes for schizophrenia and bipolar disorder exceeded that of the single loci, suggesting that the gene–gene interactions augment the probability of disease (Table 4). The TDO2/MC5R/MCHR2 genotype was associated with an OR of 4.8 for the diagnosis of schizophrenia and the HM74/MCHR1/MCHR2 risk genotype was associated with an OR of 1.7 for the combined diagnostic

category, together pertaining to approximately 40% of the patient population.

Of note, the risk alleles in HM74 and MCHR1 match those previously identified by others, i.e. the A allele in rs2454727 of HM74 (Shink et al., 2005) and the T allele in rs133073 of MCHR1 (Severinsen et al., 2006). Furthermore, the complex genotype results for the other loci are consistent with chromosomal markers previously identified as associated with schizophrenia and/or bipolar disorder in genome-wide linkage and association studies. To summarize the prior reports, significant outcomes were obtained for: a) markers within 1 cM of MCHR2 (bipolar disorder, $p \leq 0.05$, Dick et al., 2003; Lambert et al., 2005; and schizophrenia, unweighted MLS $p < 0.01$, Levinson et al., 2000); b) markers within 1 MB of MC5R (bipolar disorder, $p < 0.001$, Lin et al., 2005; schizophrenia, $p = 0.02$, Schwab et al., 1998); and c) for a broad region on chromosome 4q (133 MB–187 MB) spanning the location of TDO2 (schizophrenia, $p < 0.004$; Vawter et al., 2006) and for a marker within 1.5 MB of TDO2 (schizophrenia, $p = 0.01$, Hovatta et al., 1999).

4.2. Interpreting the genotype risks

4.2.1. Kynurenic acid as a mediator of pathophysiology

The psychotomimetic effects potentially mediated by kynurenine pathway intermediates, include antagonism of the NMDA receptor by kynurenic acid as proposed by Schwarcz et al. (2001) and reviewed by Coyle (2006). In addition, Hilmas et al. (2001) have demonstrated kynurenic

Table 4

Odds ratios, *p* values and 95% confidence intervals, from GEE models for complex genotypes derived from candidate interactions and single loci of significance, tested for association with outcome and reported by study.

Genotype	Study 1 ^a Scz OR, <i>p</i> value, (CI)	Study 2 ^b Scz OR, <i>p</i> value (CI)	Combined studies ^{a,b} , Scz OR, <i>p</i> * value (CI)	Combined studies, Scz plus bipolar disorder, OR, <i>p</i> * value, (CI)	% Scz plus bipolar disorder with genotype
<i>Complex risk genotypes, tested across study groups</i>					
A. TDO2[CC] plus MC5R[G,any] plus MCHR2[GC]	4.74, <i>p</i> = 0.044 (1.27–30.9)	5.35, <i>p</i> = 0.047 (1.02–28.0)	4.84, <i>p</i> = 0.005 (1.60–14.6)	3.93, <i>p</i> = 0.014 (1.32–11.74)	7%
B. HM74[A,any] plus MCHR1 [T,any] plus MCHR2[C,any]	2.20, <i>p</i> = 0.010 (1.18–4.24)	1.46, <i>p</i> = 0.077 (0.96–2.23)	1.69, <i>p</i> = 0.004 (1.18–2.41)	1.70, <i>p</i> = 0.003 (1.20–2.39)	30%
<i>Melanotropin receptor candidate interactions with the TDO2 or HM74 risk background</i>					
A. MC5R[G,any] plus MCHR2 [GC] in TDO2[CC]	3.74, <i>p</i> = 0.100 (0.90–25.6)	5.11, <i>p</i> = 0.084 (0.80–32.6)	4.30, <i>p</i> = 0.016 (1.31–14.14)	3.30, <i>p</i> = 0.046 (1.02–10.7)	19%
B. MCHR1 [T,any] plus MCHR2 [C,any] in HM74[A,any]	2.26, <i>p</i> = 0.027 (1.11–4.71)	1.22, <i>p</i> = 0.450 (0.73–2.05)	1.52, <i>p</i> = 0.05 (1.00–2.32)	1.52, <i>p</i> = 0.044 (1.01–2.28)	47%
<i>The single risk loci by site</i>					
HM74[A,any]	1.39, <i>p</i> = 0.270 (0.78–2.48)	1.51, <i>p</i> = 0.023 (1.06–2.15)	1.48, <i>p</i> = 0.011 (1.10–2.00)	1.50, <i>p</i> = 0.007 (1.12–2.00)	63%
TDO2[CC]	1.74, <i>p</i> = 0.077 (0.95–3.26)	1.28, <i>p</i> = 0.29 (0.81–2.00)	1.40, <i>p</i> = 0.069 (0.98–2.01)	1.42, <i>p</i> = 0.050 (1.00–2.02)	33%

^aThe SMRI sample collection. For the analyses limited to this study site, African Americans were excluded as they were too few in number in this collection.

^bThe University of Colorado Health Sciences Schizophrenia Center sample collection. Population stratification has been tested and ruled out for this sample collection (Stephens et al., 2009).

*A *p* value of 0.0083 is the threshold required for Bonferroni significance, given 6 SNP comparisons.

acid inhibition of the α -7 nicotinic receptor. Antagonism of this receptor in the rat (Shepard et al., 2003) elicits the auditory gating endophenotype associated with schizophrenia (Freedman et al., 2003). Activators of the pathway would be expected to elevate kynurenic acid levels and this may underlie the gene–gene interaction found for TDO2 and MC5R (Model 1, Fig. 1).

4.2.2. Neurotoxicity of pathway intermediates and pigment products

Neurotoxic intermediates are also of interest, as the known neurotoxins produced by kynurenic pathway activation include quinolinic acid and 3-hydroxykynurenic (Guidetti and Schwarcz, 1999). Again, these products will be generated by activation of the pathway and would potentially relate to the gene–gene interaction of two co-activators of the pathway (TDO2 and MC5R, Model 1, Fig. 1). To the list of neurotoxic products should be added the pigment products of 3-

hydroxykynurenic and 3-hydroxyanthranilic acid as the elevation in pathway intermediates extends through to 3-hydroxyanthranilic acid in schizophrenia (Miller et al., 2008). This interpretation of the pathophysiology relates to the gene–gene interaction of HM74A and MCHR1 (Model 2 in Fig. 1, wherein the pigment products may not be adequately sequestered when the risk genotype for MCHR1 is present combined with the risk genotype for HM74A, as the ligand for HM74A (niacin) controls levels of kynurenic pathway products in human subjects (Hankes et al., 1971). The types of kynurenic pathway pigments range from the xanthomattins, a form that predominates in the eye and thought to be involved in cataract formation (Vazquez et al., 2000) to the melanin family of pigments (Vogliardi et al., 2004), to the antibiotic, cinnabarinic acid (Vazquez et al., 2000). As a general class, the toxicity of pigment molecules and their reactive precursors has been well recognized, including the well-known kernicterus induced by bilirubin pigment and the toxicity of neuromelanin

Table 5

Odds ratios, confidence intervals, and *p* values for the significant variables identified by logic regression.

	Scz OR (95% CI)	<i>p</i> ^a	Scz + bipolar OR (95%CI)	<i>p</i> ^a
TDO2[CC]	1.4 (0.97–2.02)	0.07363	1.43(1.00–2.04)	0.05174
HM74[A,any]	1.48 (1.09–2.00)	0.01080	1.50(1.12–2.01)	0.00651
TDO2[CC]	2.93 (1.60–5.39)	0.00053	3.00(1.65–5.46)	0.00034
HM74[A,any]	1.91 (1.35–2.69)	0.00024	1.94(1.39–2.72)	0.00010
TDO2[CC] plus HM74[A,any]	0.31^b(0.15–0.64)	0.00143	0.31(0.15–0.62)	0.00110
TDO2[CC] or HM74[A,any]	1.96 (1.42–2.71)	0.00004	2.00(1.46–2.74)	0.00001

We compared schizophrenic subjects only to controls (columns 1–2), and schizophrenic plus bipolar subjects to controls (columns 3–4), using generalized estimation equations to account for family structure. Adjusting for potential differences in race and study sites, we fitted a main effects models (lines 1–2), a model with main effects and an interaction (lines 3–5), and a model with the Boolean term derived from logic regression (line 6).

^a Note that permutation testing with cross-validation is not confounded by multiple comparisons. It establishes the presence or absence of signal in the data, and assesses the correct model size. The *p* values are informative for quantifying the effect size relative to standard error.

^b The OR for the interaction being less than 1 means the effects of HM74[A,any] and TDO2[CC] are not additive, i.e. that adding the individual effects would significantly overestimate the odds of disease when both HM74[A,any] and TDO2[CC] are true.

when it is not adequately sequestered (Offen et al., 1997). In-vitro, the type of melanin formed can be strongly affected by the presence of the kynurenine metabolite 3-hydroxyanthranilic acid (Fig. 1), which shifts the production of the classic polymerized black eumelanin to the formation of a reddish-brown pigment that is less-completely polymerized and potentially more soluble (Soddu et al., 2004). As described for heme (Slater et al., 1991), polymerization affords protection from dispersion of the toxic component of pigment, whereas enhanced solubility increases the distance across which the toxic component can act through the cytoplasm or the inter-cellular milieu.

None of the proposed models explain the gene–gene interactions found for the melanotropin receptor MCHR2. Functional MCHR2 is expressed in humans and primates, but not rodents (Tan et al., 2002), a fact that has hindered understanding of its physiological role. In this study, genotyping of MCHR2 was undertaken solely due to its chromosomal location being associated with disease phenotype in genome-wide association studies as discussed in Section 4.1 above.

4.3. Limitations of the study

As with many genetic association and linkage studies for schizophrenia and bipolar disorder, some of the controls fall within the age range of peak onset. Here, 1/4 of the controls were less than 30 years of age at the time of diagnosis, and thus, it is certainly possible that an unknown percentage have gone on to develop a psychiatric diagnosis. However, none of the controls reported first degree relatives with schizophrenia or bipolar disorder, and thus, if some were to have been subsequently received a psychiatric diagnosis of relevance, it is likely that fewer of those cases would be genetic in origin than are cases with a family history of disease.

The actual mechanisms for the gene–gene interactions reported here cannot be ascertained from the results we present or from prior data. Therefore, three hypothetical models have been presented for the gene–gene interactions based on the known function of the genes involved, but those models are solely for the purposes of stimulating further research and do not represent established theory.

5. Conclusion

The associations and interactions we have identified in this preliminary genetic study provide the basis for a novel direction in schizophrenia and bipolar genetic research, one that is relevant to a substantial percentage of the patient population. Maximal risk for schizophrenia and bipolar disorder was found to be conveyed by a complex genotype of TDO2/MCHR2/MC5R, and, although relevant to a larger percent of the patient population, lesser risk was conveyed by a complex genotype of HM74/MCHR1/MCHR2. Obtaining the haplotype for the associated alleles will be the crucial next step for ascertaining the causative sequence and will allow a more accurate determination of risk. Follow-up studies in animal models will be necessary to decipher the mechanism of interaction between the genes for melanotropin receptors and kynurenine pathway regulators, to provide a framework for investigating interactions with other related genes, and to reveal the optimal therapeutic targets.

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Contributors

Christine L. Miller designed the study and conducted the genotyping assays. Peter Murakami conducted the statistical analyses. Ingo Ruczinski designed and supervised the statistical analyses. Randall G. Ross conducted diagnostic interviews of patients. Melissa Sinkus provided background literature relevant to understanding the interaction with the melanotropins. Bernadette Sullivan recruited and screened patients and controls; obtained consent from study participants. Sherry Leonard determined methods for and managed the acquisition of DNA samples; ascertained the proper SNPs necessary to test for population stratification.

Conflict of interest

PM, IR, RGR, MS, BS and SL have no competing financial interests. Since submission of the manuscript, CLM has transitioned to self-employment in the sole proprietorship MillerBio, a for-profit company dedicated to the development of genetic tools to study the human genome. The success of those products will be no more affected by the publication of this paper than the publication of other papers demonstrating an association between genes and human disease. Furthermore, no patents will be filed on the association between schizophrenia or bipolar disorder and the genes with the associated SNPs we report here as conveying risk for disease.

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