RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

Multivariate Analysis of Anxiety Disorders Yields Further Evidence of Linkage to Chromosomes 4q21 and 7p in Panic Disorder Families

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Received 19 August 2011; Accepted 28 December 2011

Replication has been difficult to achieve in linkage studies of psychiatric disease. Linkage studies of panic disorder have indicated regions of interest on chromosomes 1q, 2p, 2q, 3, 7, 9, 11, 12q13, 12q23, and 15. Few regions have been implicated in more than one study. We examine two samples, the Iowa (IA) and the Columba panic disorder families. We use the fuzzy-clustering method presented by Kaabi et al. [Kaabi et al. (2006); Am J Hum Genet 78: 543–553] to summarize liability to panic disorder, agoraphobia, simple phobia, and social phobia. Kaabi et al. applied this method to the Yale panic disorder linkage families and found evidence of linkage to chromosomes 4q21, 4q32, 7p, and 8. When we apply the same method to the IA families, we obtain overlapping evidence of linkage to chromosomes 4q21 and 7p. Additionally, we find evidence of linkage on chromosomes 1, 5, 6, 16, and 22. The Columbia (CO) data does not indicate linkage to any of the Kaabi et al. peaks, instead implicating chromosomes 2 and 22q11 (2 Mb from COMT). There is some evidence of overlapping linkage between the IA and CO datasets on chromosomes 1 and 14. While use of fuzzy clustering has not produced complete concordance across datasets, it has produced more than previously seen in analyses of panic disorder proper. We conclude that chromosomes 4q21 and 7p should be considered strong candidate regions for panic and fear-associated anxiety disorder loci. More generally, this suggests that analyses including multiple aspects of psychopathology may lead to greater consistency across datasets.

Key words: replication; phobia; panic; fuzzy-cluster; linkage

How to Cite this Article:
Logue MW, Bauver SR, Knowles JA, Gameroff MJ, Weissman MM, Crowe RR, Fyer AJ, Hamilton SP. 2012. Multivariate Analysis of Anxiety Disorders Yields Further Evidence of Linkage to Chromosomes 4q21 and 7p in Panic Disorder Families.
Am J Med Genet Part B 159B:274–280.

Grant sponsor: National Institute of Mental Health; Grant numbers: K01MH076100, MH2874 MH35792, 2RO1MH34728, 5KO2MH00735.
Conflict of interest: J. Knowles is on a Scientific Advisory Board for Invitrogen, Inc. and the technical advisory board of SoftGenetics, Inc. In the last 2 years M. Weissman received funding for her research from NIMH, NIDA, NARSAD, the Sackler Foundation, the Templeton Foundation, and the Interstitial Cystitis Foundation. She receives royalties from books from the American Psychiatric Association Press, Oxford Press and for an assessment from Multi Health Systems. None of the funding relates to this review. Except for NIMH support, none of the other funding relates to this review. M. Logue, S. Bauver, S. Hamilton, A. Fyer, M. Gameroff, and R. Crowe have no biomedical interests or other potential conflicts of interest to disclose.
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Published online 17 January 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/ajmg.b.32024

INTRODUCTION

Despite extensive effort studying the molecular genetics of panic disorder, no reproducible risk locus for this anxiety disorder has been established [Hamilton, 2009]. Genome-wide scans have produced evidence of linkage to multiple regions, with little overlap between the results. Table I presents a summary of peaks from the Iowa (IA), Columbia University (CO), Yale (YA), Massachusetts (MA), and Icelandic (IC) genome screens for panic disorder [Knowles et al., 1998a; Crowe et al., 2001; Gelernter et al., 2001; Smoller et al., 2001; Logue et al., 2003; Thorgeirsson et al., 2003; Fyer et al., 2006]. Lack of consensus from these studies is likely due to a combination of factors including moderate-sized samples, locus heterogeneity, and low effect sizes for common alleles in complex disease. At least some of the inability to replicate findings may be because Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria do not correspond closely to underlying genetic risk.

The concept of many anxiety and affective disorders as separate and independent entities has been undermined by the high levels of comorbidity between them [Clark et al., 1995]. In addition, population-based twin studies of the anxiety disorders and depression find evidence of broad overlapping genetic liability [Hettema et al., 2006, 2005]. Factor analysis has indicated that within the internalizing disorders, panic disorder, agoraphobia (AgP), and specific phobias are grouped together in a cluster of fear-based disorders [Krueger, 1999; Watson, 2005], while generalized anxiety disorder and major depressive disorder cluster as ''misery''-based disorders [Hettema et al., 2006]. Therefore, the examination of multiple disease states simultaneously is a reasonable, and perhaps even preferred, way to accurately summarize a subject's genetic load of risk factors within the fear-based anxiety disorders.

One attempt to do just that, was presented in Kaabi et al. [2006]. Kaabi et al. presented a multipoint scan of the YA linkage sample that combined the diagnostic status of panic disorder, simple phobia (SimP), social phobia (SocP), and AgP. Assuming that these four traits are all expression of a shared underlying fear/anxiety trait, they performed a fuzzy-cluster analysis of the subjects. This yielded a grade of membership score (GoM) quantifying the degree to which each subject fit into one of two categories corresponding to high and low anxiety disorder risk [Kaabi and Elston, 2003]. The GoM was then used as the outcome measure in a quantitative linkage analysis based on the multipoint Haseman–Elston method [Haseman and Elston, 1972]. Kaabi et al. observed empirical P-values of <0.01 across a large region on chromosome 4, with two distinct peaks ($P = 4.5 \times 10^{-3}$ on 4q21 at D4S1534 and 60 cM away $P = 5.6 \times 10^{-4}$ on 4q32 at D4S413), on chromosome 7 ($P = 1.5 \times 10^{-4}$ at D7S516), and on chromosome 8 (peak $P = 1.3 \times 10^{-3}$ at D8S277). Because the result on chromosome 4q32 is consistent across two-point and multipoint analyses and whether or not the sibling age is adjusted for the calculations, the authors focus on this locus as the most promising, noting that the theoretical P-value (which is orders of magnitude less significant than the P-value obtained by permutation) exceeds Lander and Kruglyak significance thresholds [Lander and Kruglyak, 1995]. The authors also noted that this was a novel candidate region for anxiety and that it was near the site of the neuropeptide Y receptor gene (NPY1R), which has been associated with anxiety in mouse models [Sorensen et al., 2004]. None of the empirical P-values obtained achieved a conservative Bonferroni-corrected (for 400 loci) genome-wide significance level ($P < 1.25 \times 10^{-4}$). Throughout this study we will refer to this as the Yale fuzzy-cluster analysis (YFC).

Here, we are investigating linkage in two additional linkage datasets ascertained on the basis of PD probands: the IA and the CO panic disorder pedigrees. Examination of these two datasets is especially interesting, as both have yielded evidence of linkage to chromosome 7p near the YFC peak (41.69 cM). In a genome-wide survey of the IA families, the largest maximum LOD = 2.23 was achieved on chromosome 7p at marker D7S2846 (57.79 cM according to the Marshfield genetic map) [Crowe et al., 2001]. A follow-up study [Logue et al., 2003] presented a Bayesian analysis of the same data using the posterior probability of linkage (PPL) [Vieland, 1998]. The largest PPL of 80% was observed on chromosome 7 at marker D7S521 (62 cM), indicating that there was an 80% chance that this region of chromosome 7 harbored a risk locus for PD. An earlier analysis with a subset of the ultimate CO sample indicated linkage to the same vicinity [Knowles et al., 1998a]. In an analysis based on 23 pedigrees, the largest, although still non-significant, LOD score of 1.71 was obtained at locus D7S435, 10 cM from the peak locus observed in the IA data (47.08 cM). After incorporation of 11 more pedigrees (total = 34), this score had risen to LOD = 2.45 [Knowles et al., 1998b]. However, analysis of the full 120 pedigrees of the CO data did not yield substantial evidence for linkage at this location [Fyer et al., 2006]. Even after extensive examination and follow-up genotyping, LODs and HLODs remained low across the region (maximum HLOD = 1.59). In contrast, strong evidence of linkage was obtained at several other locations, including multiple-testing corrected significant peaks on chromosome 2q (maximum HLOD = 4.19) and 15q (NPL = 3.4, empirical $P = 0.001$) and additional peaks with suggestive-level significance on chromosomes 2p (HLOD = 3.20) and 9p (HLOD = 2.98).

In this study, we perform the analogous genome-wide fuzzy-cluster linkage analysis in the IA and CO datasets. Of particular interest is whether or not the IA and CO data indicate linkage to the chromosome 7 peak from the YFC. We also examined whether or not the IA and CO data replicate the chromosome 4 and 8 peaks obtained in the YFC, neither of which has been implicated in either of these datasets previously, or if there are any novel regions of interest that are implicated in both the IA and CO datasets.

MATERIALS AND METHODS

The IA data consist of 325 individuals in 23 extended pedigrees. Pedigree sizes range from 8 to 24, with a median pedigree size of 14. The genome screen data consists of 389 microsatellite markers. Genotypes are available for 254 family members. The CO data consist of 120 multiplex PD families comprised 1,591 members. Pedigrees ranged in size from 5 to 54, with a median family size of 11. Genotyping of this sample was performed by the Center for Inherited Disease Research (http://www.cidr.jhmi.edu). Genotypes are available for 992 of the family members. The analysis here is based on the genotypes of 381 autosomal markers from the CIDR panel of microsatellite markers. While there are a few instances of overlap, this set of markers is primarily distinct from the IA dataset

panel of markers. Pedigree ascertainment, diagnosis, genotyping, and data cleaning procedures for the IA and CO data have been described in detail elsewhere ([Crowe et al., 2001] for IA, [Fyer and Weissman, 1999] for CO). The diagnoses correspond to the DSM III-R criteria [American Psychiatric Association, 1987]. In the original CO study protocol, individuals with SocP but not panic disorder were coded as affected for SocP but given a status of "unknown" for PD. This was done to allow for genetic determinants that might be shared across anxiety disorders. Here, as the Fuzzy-clustering method explicitly incorporates the possibility that PD and SocP share genetic determinants, those who are affected with SocP and unaffected with PD are simply coded as affected for SocP and unaffected for PD, which matches the Kaabi et al. diagnosis coding. For this project, the diagnoses of SimP, AgP, SocP, previously assigned as a part of both studies were re-coded into the same 0, 1, 2 ordinal scale as in YFC, with 0 indicating definitely unaffected, 1 indicating possibly or partially affected, and 2 indicating a subject was definitely affected with the particular phenotype. The frequencies of the different diagnoses in the three datasets (and the YFC as reported in Kaabi et al. [2006]) are contained in Table II.

The fuzzy-cluster analysis performed here corresponds closely to the methods presented in the YFC. The S-plus FANNY procedure (with the same default options) was used to perform the fuzzy-clustering analysis of diagnosis of panic, AgP, SimP, and SocP, with a 0, 1, 2 coding representing those unaffected, probably affected, and definitely affected, respectively. The number of clusters was fixed at 2. The resulting GoM scores rather than the cluster assignments themselves were then used as the response variable in our linkage analysis. IBD sharing for sib-pairs was computed using the GENIBD component of the S.A.G.E. package (http://darwin.cwru.edu/sage/). A multipoint linkage analysis was performed using a modified Haseman–Elston regression method [Shete et al., 2003] as implemented in S.A.G.E.'s SIBPAL component. Evidence of linkage was evaluated at each marker location. Empirical (simulation-based) *P*-values are only computed if the theoretical *P*-values exceed a threshold. We will focus our discussion on the empirical *P*-values only, as the theoretical distribution appears to differ substantially from the empirically based *P*-values. We will not be presenting single-point results, sib-pair age difference adjusted results, or the results of a heterogeneity analysis as presented in the YFC to avoid additional multiple testing penalties. We adopted the YFC convention that loci will be considered suggestive for linkage if the single-locus (uncorrected for multiple testing) *P*-value is <0.01. Genome-wide significance was judged using a conservative Bonferroni correction for the number of markers within each dataset. As the marker density was similar for both datasets we used a single genome-wide significance cutoff of $P < 1.25 \times 10^{-4}$.

RESULTS

A graph of the genome-wide results of the analysis is presented in Figure 1. Table III includes results for all markers with empirical *P*-values reaching suggestive levels of significance. No *P*-values reached genome-wide significance in either dataset.

Of the 4 regions for which suggestive empirical *P*-values were obtained in the YFC, 2 were replicated in the IA analysis. That is, suggestive evidence for linkage ($P = 8.0 \times 10^{-4}$) was obtained on chromosome 7 in the region previously shown to be linked to PD proper in the IA sample. This marker is 16 cM away from the YFC chromosome 7 peak marker. On chromosome 4, evidence of replication was obtained for the 4q21 peak. A *P*-value of 1.2×10^{-3} was obtained at D4S395, less than 3 cM away from the YFC 4q21 peak marker. No evidence of replication was seen

TABLE I. Summary of Previous PD and Phobia Genome Screens by Site of Collection

Study	Chr	Position	Marker	Score
Iowa (IA)—Crowe et al. [2001]	7	57.8	D7S2846	$LOD_{DOM,N} = 2.2$
Columbia (CO)—Knowles et al. [1998a]	7	47.1	MFD20	$LOD_{REC,2P} = 1.7$
CO—Knowles et al. [1998b]	7	47.1	MFD20	$LOD_{REC,2P} = 2.5$
CO—Fyer et al. [2006]	2p	55.5	D2S1788	$HLOD_{DOM,2P,SS,I} = 3.7$
	2q	260.6	D2S125	$HLOD_{DOM,2P,SS,B} = 4.2$
	9	32.2	D9S925	$HLOD_{REC,2P,SS,I} = 3.2$
	12	109.5	PAH	$LOD_{DOM,2P,SS,B} = 2.6$
	15	12.3	D15S822	$NPL_{MP,B} = 3.4$
Yale (YA)—Gelernter et al. [2001]	1q	258.4	D1S2785	$LOD_{DOM,MP,B} = 2.0$
	3	167.5	D3S1279	$NPL_{MP,A} = 2.8$
	11	5.3	CCKBR	$LOD_{DOM,MP,B} = 2.0$
YA—Gelernter et al. [2003]	14	36.7	D14S75	$LOD_{DOM,MP,SimP} = 3.7$
YA—Gelernter et al. [2004]	16		D16S415	$Zlr_{MP,SocP} = 3.41$
Iceland (IC)—Thorgeirsson et al. [2003]	9	109.9	D9S271	$AO\text{-}LOD_{MP,PD+ANX} = 4.2$
Massachusetts (MA)—Smoller et al. [2001]	10	147.6	D10S587	$LOD_{D,MP,DTa} = 2.4$
	12	66.0	D12S368	$NPL_{MP,PD+A} = 5.0$

DOM, dominant genetic model; REC, recessive genetic model; 2P, two-point analysis; MP, multi-point analysis; N, narrow panic disorder diagnostic criteria; I, intermediate panic disorder diagnostic criteria; B, broad panic disorder diagnostic criteria; SS, sex-specific recombination fractions; A, agoraphobia only diagnostic criteria; PD + ANX, panic disorder + anxiety disorder; DT_a, best-estimate diathesis affection type; PD + A, panic disorder and agoraphobia phenotype; SimP, simple phobia; SocP, social phobia.

TABLE II. Frequency (%)of Anxiety Phenotypes Among Subjects

Phenotype	Definitely Affected (2)			Probably or partially affected (1)			Definitely unaffected (0)			Missing or unknown		
	CO	IA	YFC[a]	CO	IA	YFC[a]	CO	IA	YFC[a]	CO	IA	YFC[a]
Agoraphobia	424 (26)	75 (23)	76 (38)	1 (<1)	9 (3)	4 (2)	1235 (74)	214 (66)	115 (57.5)	0	27 (8)	5 (2.5)
Simple phobia	290 (17.5)	20 (6)	74 (37)	39 (2.3)	3 (<1)	5 (2.5)	1331 (80.2)	275 (85)	116 (58)	0	27 (8)	5 (2.5)
Social phobia	264 (16)	20 (6)	65 (32.5)	29 (2)	3 (<1)	5 (2.5)	1367 (82)	275 (85)	125 (62.5)	0	27 (8)	5 (2.5)
Panic disorder	606 (36)	89 (27)	55 (27.5)	197 (12)	31 (10)	7 (3.5)	248 (15)	177 (54)	133 (66.5)	609 (37)	28 (9)	5 (2.5)

[a]As reported by Kaabi et al. [2006].

for the 4q32 peak or the chromosome 8 peak. None of the ROI from the YFC were replicated in the CO dataset.

Outside of the previously implicated regions, both the IA and CO data had peaks that neared genome-wide significance. In the IA data, the most significant *P*-value of 2.0×10^{-4} was observed on chromosome 5q35 at marker D5S408 (180.0 Mb). This marker is 5.1 Mb away from the dopamine receptor D1 (*DRD1*) gene. A polymorphism in DRD1 was marginally significantly associated ($P = 0.02$) with PD in a study of 90 candidate gene polymorphisms within the pure-PD (no comorbidity) subgroup [Maron et al., 2005]. Additional markers with "suggestive" evidence of linkage were observed on chromosomes 1p, 6, 16, and 17. The most significant evidence of linkage in the CO data occurred on chromosome 2q at D2S2585 ($P = 6.0 \times 10^{-4}$ at 263.56), with another suggestive *P*-value 11 cM away at marker D2S2968 ($P = 1.0 \times 10^{-2}$ at 251.94 cM). These markers lie within the chromosome 2q region previously identified as linked to PD using these data. The only other region achieving the "suggestive" level of significance in the CO data was chromosome 22q at marker D22S420 (4.06 cM). This marker is 2.1 Mb away from the catechol-*O*-methyltransferase (*COMT*) gene, which had been previously associated and linked to PD in a subset of 70 pedigrees from this dataset [Hamilton et al., 2002].

We also observed several regions where nominally significant ($P < 0.05$) peaks corresponded within the IA and CO data. On chromosome 1p12, marker D1S534 had an empirical *P*-value of 3.4×10^{-3} in the IA data. A *P*-value of 0.015 was observed at this same marker in the CO data—one of the few instances where the same marker was genotyped across both datasets. Another area with overlapping evidence of linkage was on chromosome 14. A *P*-value of 0.011 on chromosome 14 at marker D14S608 (28.01 cM) in the CO data was matched by a similar *P*-value of 0.018 at marker D14S297 (31.75 cM) in the IA data. These were the only nominally significant markers observed on chromosome 14 from either dataset.

DISCUSSION

We have duplicated the analysis presented in a multivariate examination of panic and phobia as in the YFC in two additional datasets consisting of multiplex pedigrees ascertained for the presence of PD. We used fuzzy-clustering techniques, not to generate distinct clusters of individuals, but as a multidimensional reduction technique, to compute a score summarizing the presence or absence of panic disorder, SocP, SimP, and AgP. While the YFC paper had obtained peaks on chromosomes 4q21, 4q32, 7p, and 8, they focused the discussion on the chromosome 4q32 peak due to prior evidence of the role of neuropeptide Y's involvement in anxiety from animal models. While we agree that neuropeptide Y is an interesting candidate locus for a variety of reasons, we do not find any evidence to support the role of this region in anxiety disorder susceptibility in either the IA or CO datasets.

In the IA data, 2 of the 4 regions originally implicated in the YFC were replicated: namely 4q21 and 7p. We point out that while these markers did not meet genome-wide corrected criteria for significance, they would be significant if we had only examined the markers in the region of the YFC peaks and focused solely on replicating the previous loci, rather than utilizing a genome-wide

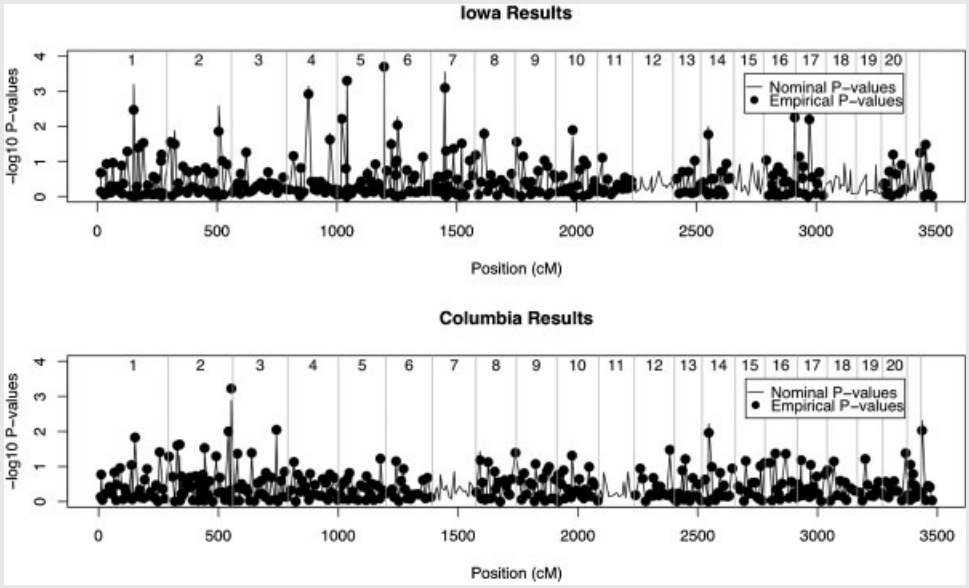

FIG. 1. Multivariate scan for linkage using GoM scores to summarize panic disorder and phobia susceptibility.

study design. Chromosome 7p is the location of the neuropeptide S receptor gene (*NPSR1*), which has been implicated in genetic association studies of panic disorder [Domschke et al., 2010]. To our knowledge, there has been no previously studied putative panic or anxiety gene on chromosome 4q21.

None of the YFC regions were replicated in the CO data. This may be due to locus heterogeneity, or differences in the distribution of PD diagnosis (Table II). In particular, there is a much higher rate of those classified as unknown with respect to PD diagnosis (PD-unknown) in the CO data (37%) than the IA or YA data (9% and 2.5%, respectively). Data collection in the CO data included a very stringent diagnostic criteria designed to minimize misclassification. This included collection of interview, family history, narrative summaries, and medical records. Final diagnosis was arrived at by senior clinicians using both best-estimate and consensus methodology. Subjects were classified as PD-unknown if they did not fit neatly into one of the other categories (definitely affected, probably affected, or unaffected). This included individuals which had an ambiguous clinical picture or with limited information from relatives which prevented a definitive diagnosis. This higher rate of PD-unknown diagnosis might have caused a difference in the GoM score between the different datasets, as GoM scores summarize the variance within a dataset. We explored the effect of the higher rate of individuals classified as unknown with respect to PD diagnosis (PD-unknown) by re-analyzing the CO data with all of those coded as PD-unknown reclassified as PD-unaffected. This analysis did not yield any evidence of replication of the YFC peaks. No *P*-values <0.01 were observed using this modified phenotype on chromosomes 4 and 7. Only one *P*-value in the suggestive level of significance was observed on chromosomes 8 ($P = 0.0074$ at a marker 100 cM away from the YFC linkage region). However, this is not conclusive evidence that the distribution of diagnoses is not the source of the absence of a peak on those chromosomes, as the blanket assignment of PD-unknown individuals as PD-unaffected likely induced diagnostic misclassification, which could reduce power to detect linkage.

Nevertheless, the replication of 2 of the 4 YFC implicated regions in the IA dataset is encouraging as replication from linkage studies of panic disorder has been elusive. Therefore, chromosome 4q21 and chromosome 7p should be considered promising candidate regions for panic and fear susceptibility.

We further observed evidence of overlapping linkage between the IA and CO data on chromosomes 1p and 14, with nominally significant *P*-values observed in close proximity from each dataset. Again, to our knowledge these are not regions previously examined for association to panic or fear. (The chromosome 1p peak is over 100 cM from the chromosome 1q peak reported in the YA families in linkage studies of panic and PD [Gelernter et al., 2001].) As none of these *P*-values reached genome-wide level significance and only the IA chromosome 1 marker reached the suggestive level, this correspondence between linkage signals should be interpreted with caution.

Additionally, there are several dataset-specific peaks. The 2q peak observed in the CO data had been implicated in an analysis of PD [Fyer et al., 2006]. The chromosome 22q peak is 2.1 Mb away from the *COMT* gene. *COMT* has previously been examined in a subset of the CO data [Hamilton et al., 2002]. That analysis yielded evidence of linkage and association to a microsatellite upstream of COMT (HLOD = 2.93, association $P = 0.001$ at D22S944). In contrast, the peaks on chromosomes 5, 6, 16, and 17 observed in the IA data were not seen in previous univariate analyses of PD. The chromosome 5q35 peak, the most significant observed in the IA data, is in the vicinity of *DRD1*. *DRD1* has been previously associated with PD [Maron et al., 2005] in a candidate gene study of multiple neurotransmitter-related genes. *DRD1* has also been implicated in studies of bipolar [Severino et al., 2005;

TABLE III. Fuzzy-Cluster Analysis Results: Markers Showing Possible Linkage ($P < 0.01$)

Chromosome and marshfield map position (cM)	Marker	Nominal *P*	Empirical *P*
IA			
Chromosome 1			
151.88	D1S534	6.4×10^{-4}	3.4×10^{-3}
Chromosome 2			
Chromosome 4			
92.42	D4S395	7.2×10^{-3}	1.2×10^{-3}
Chromosome 5			
19.02	D5S807	5.7×10^{-3}	6.1×10^{-3}
41.06	D5S819	5.2×10^{-4}	5.0×10^{-4}
195.49	D5S408	2.8×10^{-4}	2.0×10^{-4}
Chromosome 6			
53.82	D6S2427	5.3×10^{-3}	9.2×10^{-3}
Chromosome 7			
57.8	D7S2846	2.8×10^{-4}	8.0×10^{-4}
Chromosome 16			
130.41	GATA71F09	7.0×10^{-3}	5.6×10^{-3}
Chromosome 17			
56.48	D17S1293	3.6×10^{-3}	6.4×10^{-3}
Chromosome 22			
CO			
Chromosome 1			
Chromosome 2			
251.94	D2S2968	8.7×10^{-3}	1.0×10^{-2}
263.56	D2S2585	1.3×10^{-3}	6.0×10^{-4}
Chromosome 4			
Chromosome 5			
Chromosome 6			
Chromosome 7			
Chromosome 16			
Chromosome 17			
Chromosome 22			
4.06	D22S420	4.9×10^{-3}	9.4×10^{-3}

Dmitrzak-Weglarz et al., 2006], blood pressure/hypertension [Sato et al., 2000; Lu et al., 2006], schizophrenia [Allen et al., 2008], and ADHD [Misener et al., 2004; Bobb et al., 2005; Luca et al., 2007].

As both the IA and CO samples were ascertained for the presence of panic disorder, further work is needed to determine whether or not putative risk loci are linked to risk of phobia in panic-free families or whether or not these loci increase the risk to additional psychiatric disorders. Additionally, the software used does not provide the weights accorded to each of the diagnoses in the process, which makes interpretation of the GoM score and comparing across the different datasets problematic. We note that examination of the GoM scores generally follow our preconceived notions of a degree of severity measure in both datasets. That is, a diagnosis of AgP causes a larger shift in the GoM score than a diagnosis of specific or SocP and each additional diagnosis increases an individual's score. However, as the GoM score maximizes the discriminatory power within ascertained families, further research is necessary before concluding that the score would be useful or optimal in characterizing unselected samples. However, these results do suggest that lack of replication in panic disorder research has at least in part been due to the over-reliance on strict DSM criteria rather than more basic characterizations of fear and anxiety. This study also lends support for the molecular investigation of the underlying circuitry of fear in the study of panic disorders and anxiety. This would include genes identified as affecting fear response and conditioning in mice, and biomarkers of fear and emotional processing in humans [e.g., Etkin and Wager, 2007; Park et al., 2011].

ACKNOWLEDGMENTS

This work was supported by funding from the National Institute of Mental Health (Grant Nos. K01MH076100 (M.W.L.), MH2874 (M.M.W.), MH35792 (A.J.F.), and 2RO1MH34728 and 5KO2MH00735 (R.R.C.). Genotyping services were provided to J.A.K. by the Center for Inherited Disease Research, which is fully funded through Federal Contract N01-HG-65403 from the National Institutes of Health to The Johns Hopkins University. Computation was performed on the LINGA II cluster funded by a Boston University Department of Medicine Grant (Anita L. DeStefano and Emelia Benjamin, Co-PIs). The results of this study were obtained by using the program package S.A.G.E., which is supported by a U.S. Public Health Service Resource Grant (RR03655) from the National Center for Research Resources.

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