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Identification of Significant Association and Gene-Gene Interaction of GABA Receptor Subunit Genes in Autism

D. Q. Ma,[1] P. L. Whitehead,[1] M. M. Menold,[1] E. R. Martin,[1] A. E. Ashley-Koch,[1] H. Mei,[3] M. D. Ritchie,[4] G. R. DeLong,[2] R. K. Abramson,[5] H. H. Wright,[5] M. L. Cuccaro,[1] J. P. Hussman,[6] J. R. Gilbert,[1] and M. A. Pericak-Vance[1]

[1]Center for Human Genetics and [2]Division of Pediatric Neurology, Duke University Medical Center, Durham, NC; [3]North Carolina State University, Raleigh; [4]Center for Human Genetics Research, Vanderbilt University, Nashville, TN; [5]University of South Carolina School of Medicine, Columbia; and [6]The Hussman Foundation, Ellicott City, MD

Autism is a common neurodevelopmental disorder with a significant genetic component. Existing research suggests that multiple genes contribute to autism and that epigenetic effects or gene-gene interactions are likely contributors to autism risk. However, these effects have not yet been identified. Gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the adult brain, has been implicated in autism etiology. Fourteen known autosomal GABA receptor subunit genes were studied to look for the genes associated with autism and their possible interactions. Single-nucleotide polymorphisms (SNPs) were screened in the following genes: *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1* on chromosome 4p12; *GABRB2*, *GABRA6*, *GABRA1*, *GABRG2*, and *GABRP* on 5q34-q35.1; *GABRR1* and *GABRR2* on 6q15; and *GABRA5*, *GABRB3*, and *GABRG3* on 15q12. Intronic and/or silent mutation SNPs within each gene were analyzed in 470 white families with autism. Initially, SNPs were used in a family-based study for allelic association analysis—with the pedigree disequilibrium test and the family-based association test—and for genotypic and haplotypic association analysis—with the genotype-pedigree disequilibrium test (geno-PDT), the association in the presence of linkage (APL) test, and the haplotype family-based association test. Next, with the use of five refined independent marker sets, extended multifactor-dimensionality reduction (EMDR) analysis was employed to identify the models with locus joint effects, and interaction was further verified by conditional logistic regression. Significant allelic association was found for markers *RS1912960* (in *GABRA4*; $P = .01$) and *HCV9866022* (in *GABRR2*; $P = .04$). The geno-PDT found significant genotypic association for *HCV8262334* (in *GABRA2*), *RS1912960* and *RS2280073* (in *GABRA4*), and *RS2617503* and *RS12187676* (in *GABRB2*). Consistent with the allelic and genotypic association results, EMDR confirmed the main effect at *RS1912960* (in *GABRA4*). EMDR also identified a significant two-locus gene-gene effect model involving *RS1912960* in *GABRA4* and *RS2351299* in *GABRB1*. Further support for this two-locus model came from both the multilocus geno-PDT and the APL test, which indicated a common genotype and haplotype combination positively associated with disease. Finally, these results were also consistent with the results from the conditional logistic regression, which confirmed the interaction between *GABRA4* and *GABRB1* (odds ratio = 2.9 for interaction term; $P = .002$). Through the convergence of all analyses, we conclude that *GABRA4* is involved in the etiology of autism and potentially increases autism risk through interaction with *GABRB1*. These results support the hypothesis that GABA receptor subunit genes are involved in autism, most likely via complex gene-gene interactions.

Introduction

Autistic disorder (MIM 209850) is a neurodevelopmental disorder characterized by impairments in reciprocal social interaction and communication and the presence of restricted and repetitive patterns of interest or behavior. These impairments are apparent in the first 3 years of life and persist into adulthood. With the improved detection and recognition of autism that has resulted from a broadening of the diagnostic concept and systematic population approaches, a recent prevalence study reported that autistic disorder affects as many as 1 in 300 children in a U.S. metropolitan area (Yeargin-Allsopp et al. 2003). The increase in prevalence has drawn significant attention from scientists, and a rapid increase in the level of interest in the etiology of autism has been seen in the past decade (Fombonne 1999, 2003).

Autism has turned out to be one of the most heritable complex genetic disorders in psychiatry. A strong genetic component in autism is indicated by an increased

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Address for correspondence and reprints: Dr. Margaret Pericak-Vance, Center for Human Genetics, DUMC Box 3445, 595 LaSalle Street, Durham, NC 27710. E-mail: mpericak.vance@duke.edu

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concordance rate in MZ twins (60% and 91% for the narrow and broad phenotypes, respectively), compared with that in DZ twins (0% and 10% for the narrow and broad phenotypes, respectively) (Steffenburg et al. 1989; Bailey et al. 1995), and by a 75-fold greater risk to siblings of idiopathic cases, in comparison with the risk to the general population (Bolton et al. 1994). Collectively, these studies suggest that autistic disorder involves multiple variants in multiple unlinked loci that interact to cause the autism phenotype. In addition to genetic risk-assessment studies, both direct mapping approaches (chromosomal methods and linkage and association studies) and indirect mapping approaches (the characterization of disorders that share some of the symptoms of autism, such as Rett or fragile X syndrome) have been applied to identify autism-susceptibility genes. These studies have also yielded convincing evidence for the multigenic inheritance and the locus or allelic heterogeneity in autism.

Over 10 genomewide autism screens have been performed (International Molecular Genetic Study of Autism Consortium 1998, 2001; Meyers et al. 1998; Philippe et al. 1999; Risch et al. 1999; Collaborative Linkage Study of Autism 2001; Liu et al. 2001; Auranen et al. 2002; Shao et al. 2002; Yonan et al. 2003). Results from these various screens indicate potential susceptibility genes spread across the entire genome. Estimates of the number of genes involved in autism range from 3–10 (Pickles et al. 1995; Folstein and Rosen-Sheidley 2001) to $\geqslant$15 (Risch et al. 1999) to 100 (Pritchard 2001). Numerous association studies of the candidate genes have been conducted on the basis of location in a linkage peak or potential function, but no single gene has been consistently replicated across studies. One explanation for the lack of consistency in association studies is that there are many contributing genetic and environmental factors in autism. Moreover, multiple interacting genes may be the main causative determinants of autism (Muhle et al. 2004; Veenstra-VanderWeele et al. 2004). With only a modest sample size, a small-to-moderate locus effect is not easily detected. Therefore, tests for joint effects may be more successful in the search for autism-susceptibility genes.

Several lines of research indicate that there are abnormalities in the gamma-aminobutyric acid (GABA) system that may lead to developmental changes similar to those observed in autism. The evidence implicates GABA receptor (GABAR) subunit genes as functional candidates for autism (Blatt et al. 2001; Hussman 2001; Aldred et al. 2003). GABA acts on the GABAR complex, a heteromeric structure, and mediates synaptic inhibition in the adult brain (Hahn et al. 2003; Moore 2003). During development, GABA also acts as an excitatory neurotransmitter because of the high intracellular chloride concentration in immature neurons (Jentsch et al. 2002). Eight GABA classes (α, β, δ, ε, γ, π, θ, and ρ) and 18 receptor subunit genes have been characterized in mammals. In addition to providing binding sites for GABA, the GABAR contains sites for several therapeutic agents, including benzodiazepines, barbiturates, anesthetics, and alcohols. Binding studies that used labeled ligands in children indicate that GABAR density is greater early in life and then dramatically decreases to adult levels (Chugani et al. 2001). Subunit composition varies developmentally and across brain structure. It is notable that the studies found a significant decrease in GABAR density in autism (Blatt et al. 2001) and an elevated plasma GABA level in autistic children (Dhossche et al. 2002).

The most promising region identified by autism association studies is on chromosome 15q12, which harbors a set of three GABAR subunit genes (Cook et al. 1998; Martin et al. 2000*a*; Wolpert et al. 2000; Boyar et al. 2001; Menold et al. 2001; Buxbaum et al. 2002). Chromosome 15q11-q13 duplications and deletions have also been documented in children with autism (Bundey et al. 1994; Smith et al. 2000; Pujana et al. 2002). In addition, several groups have identified this region as an area of interest through linkage studies (Philippe et al. 1999; Liu et al. 2001). Follow-up fine mapping narrowed this 15q region to the *GABRB3* gene by use of a phenotypic subtype defined by a high degree of insistence on sameness (Shao et al. 2003). All of these findings from direct or indirect mapping studies strongly suggest that the GABAR subunit genes may play an important role, both independently and interactively, in the etiology of autism.

Epistasis or gene-gene interaction has been widely accepted as an important contributor to the complexity of mapping complex disease genes (Moore 2003). The failure to replicate some single-locus results might be the result of an underlying genetic architecture in which gene-gene interactions are the norm rather than the exception (Moore and Williams 2002). Thus, genetic studies that ignore epistasis or gene-gene interactions are likely to reveal only part of the genetic architecture. Although the term "epistasis" was initially used by William Bateson early in the 20th century to describe the reason for distortions of Mendelian segregation ratios and was later defined by Fisher as deviation from additivity in a linear statistical model (Moore 2005), the methodology for testing epistasis or gene-gene interaction is still in its infancy. The available methods have been thoroughly reviewed recently (Thornton-Wells et al. 2004). In general, a lack of powerful statistical methods and large sample sizes limits the identification and characterization of gene-gene interactions (Moore and Williams 2002). The main issues confronted by traditional methods, such as logistic regression, are insufficient power and flexibility to detect high-order gene-

Table 1

The PDT and geno-PDT Association Analyses of GABA Genes and Autism

Chromosome, Gene, and SNP	Marker Number	Global *P* for[a]	
		PDT[b]	geno-PDT[c]
Chromosome 4:			
GABRG1:			
RS1497571	1	.899	.906
RS2350439	2	.509	.717
RS1826923	3	.340	.622
GABRA2:			
HCV7537166	4	.556	.778
RS279858	5	.361	.623
RS279844	6	.138	.294
HCV8262290	7	.064	.141
RS4695152	8	.508	.730
HCV8262334	9	.149	***.033***
GABRA4:			
RS7678338	10	.737	.936
RS1512136	11	.935	.996
HCV1592545	12	1.000	.968
RS1912960	13	***.012***	***.003***
RS2280073	14	.072	***.034***
RS10517174	15	.738	.142
RS3792211	16	.677	.391
GABRB1:			
RS2351299	17	.817	.098
RS1372496	18	.088	.160
RS3114084	19	.180	.317
HCV11353524	20	.115	.243
HCV2119841	21	.906	.432
RS6289	22	.544	.276
RS6290	23	.940	.506
Chromosome 5:			
GABRB2:			
RS253017	24	.774	.317
RS252965	25	.649	.299
RS2617503	26	.108	***.025***
RS2962425	27	.367	.443
RS2962407	28	.771	.149
RS12187676	29	.407	***.015***
GABRA6:			
RS3811995	30	.613	.488
RS6883829	31	.932	.236
HCV164095	32	.814	.920
RS3811991	33	.652	.283
GABRA1:			
RS4340950	34	.426	.650
HCV11258504	35	.633	.601
RS6878494	36	.395	.699
HCV1667770	37	.861	.522
HCV11814555	38	.294	.576
GABRG2:			
RS7728001	39	.670	.833
RS766349	40	.700	.223
RS211014	41	.655	.815
GABRP:			
HCV3165046	42	.872	.779
RS1812910	43	.965	.981
RS1862242	44	.347	.593
RS1063310	45	.560	.807
Chromosome 6:			
GABRR1:			
RS404943	46	.619	.674
RS407206	47	.623	.835
RS423463	48	.475	.718
RS3777530	49	.644	.831
RS2297389	50	.851	.150
RS881293	51	.832	.978
RS6902106	52	.829	.712
GABRR2:			
RS282117	53	.277	.494
HCV9866022	54	.064	.171
RS2148174	55	.855	.770
HCV9865968	56	.780	.962
Chromosome 15:			
GABRB3:			
RS2081648	57	.602	.837
RS1426217	58	.191	.305
RS754185	59	.672	.852
HCV8865209	60	.337	.521
RS2059574	61	.304	.405
GABRA5:			
HCV42974	62	.646	.072
RS7173260	63	.938	.845
RS140681	64	.886	.762
RS140683	65	.825	.978
GABRG3:			
HCV2078506	66	.079	.240
RS208129	67	.281	.266
RS897173	68	.240	.451
HCV428306	69	1.000	.611
RS140679	70	.410	.589

[a] *P* values <.05 are shown in bold italics.
[b] *P* values adjusted for two alleles.
[c] *P* values adjusted for three genotypes.

gene interactions. Several newly developed methods, such as the multilocus genotype-pedigree disequilibrium test (geno-PDT) (Martin et al. 2003*a*) and the multifactor-dimensionality reduction (MDR) method (Ritchie et al. 2001), improve the ability to identify the high-order gene-gene interactions with the use of relatively small sample sizes. However, the methods have difficulty in distinguishing true interactive effects from joint effects. With the data-driven analytic methods that are continually in development to examine complex genetic interactions, it has become increasingly important to emphasize model validation to ensure that significant effects represent true relationships rather than chance findings (Coffey et al. 2004). Thus, a multianalytic approach to the analysis of gene-gene interactions was proposed (Ashley-Koch et al. 2004), an approach that searches for consistency of results and preponderance of evidence to draw the most useful conclusions. In the

Table 2

Best Gene-Gene Effect Models Identified by EMDR for GABAR Subunit Genes on Chromosome 4

Gene and Marker	Marker Number	Location	Best Model	Nonfixed *P* Value[a]	
				χ^2	Misclassification
GABRG1:					
RS1497571	1	65.47	9	.06	.038
RS2350439	2	65.49	9, 13	.004	***.002***[b]
GABRA2:			2, 9, 15	.016	.03
RS279858	3	65.66	2, 7, 9, 15	.16	.33
RS279844	4	65.66			
HCV8262290	5	65.67			
RS4695152	6	65.68			
HCV8262334	7	65.68			
GABRA4:					
HCV1592545	8	65.85			
RS1912960	9	65.86			
RS2280073	10	65.86			
RS10517174	11	65.87			
RS3792211	12	65.87			
GABRB1:					
RS2351299	13	65.92			
RS1372496	14	65.94			
RS3114084	15	65.95			
HCV11353524	16	65.97			
HCV2119841	17	65.99			
RS6289	18	66.00			
RS6290	19	66.00			

[a] Empirical *P* value derived from nonfixed permutation test by use of χ^2 or the misclassification rate as the test statistic in EMDR.

[b] The locus with the lowest *P* value (in bold italics) was selected as the one for the final cross-chromosome model.

current study, this new paradigm was applied to test our hypothesis that GABAR subunit genes may contribute to the etiology of autism independently and/or through complex interactions between subunit genes.

Families and Methods

Family Ascertainment

A standard ascertainment protocol was conducted by the clinical groups at the Duke Center for Human Genetics and the William S. Hall Psychiatric Institute. Both sites recruited, enrolled, and sampled individuals with autism and family members per study protocols approved by their respective institutional review boards (IRBs). Participating families were ascertained using clinical referrals and active recruitment through lay organizations that provide services to families with autism. After a full description of the study was given to the families, written informed consent was obtained from parents and from children who were able to give informed consent. For the current study, a total of 470 white families were included, of which 266 were multiplex (i.e., more than one affected individual sampled) and 204 were triads (i.e., only one affected individual sampled). The Collaborative Autism Team from the Duke Center for Human Genetics and the William S. Hall Psychiatric Institute contributed 246 families, and 224 families were from the Autism Genetic Resource Exchange. Probands for the study consisted of individuals between the ages of 3 years and 21 years who received a clinical diagnosis of autism by use of DSM-IV criteria. A consistent set of diagnostic criteria was applied to all families. Qualified individuals and families were those who met best-estimate clinical research diagnoses for autism, as determined by the lead clinicians (H.H.W. and M.L.C.) at each of the research sites. The best-estimate diagnoses were made using all available case material, including clinical records, Autism Diagnostic Interview–Revised (ADI-R) results, and clinical assessment information. All qualified individuals met current DSM-IV diagnostic criteria for autism. The ADI-R (Lord et al. 1997) is a validated, semistructured diagnostic interview, which yields a diagnostic algorithm based on the DSM-IV criteria for autism. All ADI-R interviews were conducted by formally trained interviewers who have established reliability within our group as well as within Dr. Lord's group. Finally, all participants who met current diagnostic criteria for au-

Table 3

Best Gene-Gene Effect Models Identified by EMDR for GABAR Subunit Genes on Chromosome 5

Genes and Marker	Marker Number	Location	Best Model	Nonfixed *P* Value[a]	
				χ^2	Misclassification
GABRB2:					
RS253017	1	165.09	6	.412	***.282***[b]
RS252965	2	165.09	3, 6	.657	.762
RS2617503	3	165.098	4, 8, 18	.710	.551
RS2962425	4	165.155	3, 10, 13, 17	.810	.800
RS2962407	5	165.203			
RS12187676	6	165.246			
GABRA6:					
HCV164095	7	165.362			
RS3811991	8	165.364			
GABRA1:					
RS4340950	9	165.476			
RS6878494	10	165.494			
HCV1667770	11	165.502			
HCV11814555	12	165.505			
GABRG2:					
RS169793	13	165.667			
RS7728001	14	165.674			
RS766349	15	165.677			
RS211014	16	165.693			
GABRP:					
HCV3165046	17	182.846			
RS1812910	18	182.859			
RS1862242	19	182.871			
RS1063310	20	182.877			

[a] Empirical *P* value derived from nonfixed permutation test by use of χ^2 or the misclassification rate as the test statistic in EMDR.

[b] The locus with the lowest *P* value (in bold italics) was selected as the one for the final cross-chromosome model.

tism were included only if they had a minimal developmental level of 18 mo for the Vineland Adaptive Behavior Scale score (Sparrow et al. 1984) or had an IQ equivalent >35. These minimal developmental levels assure that ADI-R results are valid and reduce the likelihood of including individuals with severe mental retardation only. Subjects were excluded if they had evidence of developmental disorders with known phenotypic overlap with autism (e.g., Prader-Willi syndrome, Angelman syndrome, tuberous sclerosis complex, Rett syndrome, and fragile X syndrome), neurologic disorders, or severe sensory or motor disorders.

Genotyping

Blood was obtained from patients and other family members in accordance with IRB-approved procedures. DNA was extracted from whole blood by use of standard protocols (Vance 1998). Analysis of the candidate region was performed using data obtained from SNPs. SNPs located within the GABAR subunit genes across chromosomes were analyzed. Between three and seven intronic and silent mutation SNPs within each gene were identified with Applied Biosystems (ABI) Assay-on-Demand products. The selected GABAR subunit genes were *GABRG1* (3 SNPs typed), *GABRA2* (6), *GABRA4* (7), and *GABRB1* (7) on 4p12; *GABRB2* (6), *GABRA6* (4), *GABRA1* (5), *GABRG2* (3), and *GABRP* (4) on 5q34-q35.1; *GABRR1* (7) and *GABRR2* (4) on 6q15; and *GABRB3* (5), *GABRA5* (4), and *GABRG3* (5) on 15q12. SNPs were identified from the National Center for Biotechnology Information SNP database (see dbSNP Web site) and were ordered for use in either Assays-on-Demand or Assays-by-Design (ABI). All SNPs were genotyped using TaqMan. All reactions contained 2.7 ng of total genomic DNA and were run on ABI 9700 GeneAmp PCR systems in accordance with the manufacturer's instructions. Analysis of the SNP genotypes was performed using an ABI Prism 7900HT Sequence Detection System.

For quality control (QC) procedures, two CEPH standards were included on each 96-well plate, and samples from six individuals were duplicated across all plates as QCs, with the laboratory technicians blinded to their identities. Analysis required that identical QC samples within and across plates have matching genotypes, to identify errors in loading and reading and thus to min-

Table 4

Best Gene-Gene Effect Models Identified by EMDR for GABAR Subunit Genes on Chromosome 6

Genes and Marker	Marker Number	Location	Best Model	Nonfixed *P* Value[a]	
				χ^2	Misclassification
GABRR1:					
RS404943	1	94.926	8	.644	.587
RS407206	2	94.935	5, 11	.579	.452
RS423463	3	94.946	2, 8, 10	.398	***.246***[b]
RS3777530	4	94.975	7, 8, 10, 11	.645	.665
RS2297389	5	94.991			
RS881293	6	94.998			
RS6902106	7	95.019			
GABRR2:					
RS282117	8	95.171			
HCV9866022	9	95.208			
RS2148174	10	95.238			
HCV9865968	11	95.277			

[a] Empirical *P* value derived from nonfixed permutation test by use of χ^2 or the misclassification rate as the test statistic in EMDR.

[b] The locus with the lowest *P* value (in bold italics) was selected as the one for the final cross-chromosome model.

imize the error rate in genotype assignments. Meanwhile, a 95% efficiency of genotype is required. Technicians generating the genotypic data were blinded to the clinical status of the patients. After QC verification, genotypes of the samples were uploaded into the PEDIGENE database and were merged into the LAPIS management system for creation of analysis input files (Haynes et al. 1995).

Statistical Analysis

To further check for genotyping error, the program PedCheck was run for detection of Mendelian inheritance inconsistency. The error checking option embedded in Merlin (Abecasis et al. 2002) was run to identify the samples with excess recombinations, and the families were checked further for possible genotyping errors. One affected and one unaffected individual were selected randomly from each family for tests of Hardy-Weinberg equilibrium (HWE), which was assessed using exact tests implemented in the Genetic Data Analysis program (Zaykin et al. 1995). For SNPs found to be not in HWE in the unaffected sample, a sequence of samples at that particular SNP was required to ensure the quality of the SNPs. Pairwise linkage disequilibrium (LD) (D' and r^2) between markers was calculated using the GOLD software package (Abecasis and Cookson 2000). The allelic association analyses were conducted using the pedigree disequilibrium test (PDT) (Martin et al. 2000*b*) and the family-based association test (FBAT) (Horvath et al. 2004). These two tests are similar in many aspects, but each has distinct advantages. The PDT has the advantage of being valid as a test of both linkage and association in extended pedigrees, whereas the FBAT treats nuclear families within large pedigrees as independent but permits haplotype-based association tests. Both PDT and FBAT are allele-based tests. The geno-PDT (Martin et al. 2003*a*) is an extension of PDT used to examine the association between marker genotype and disease. The haplotype family-based association test (HBAT) (Horvath et al. 2004) was used for haplotype association analysis for SNPs within each GABAR subunit gene. Tagging SNPs within each gene were selected by use of the confidence-interval function in Haploview (Barrett et al. 2005). Both haplotype-specific *P* values and global *P* values (with adjustment for all possible haplotypes) were calculated by the program.

The core program of MDR (Ritchie et al. 2001; Hahn et al. 2003) was employed in this study to test for potential gene-gene interaction and thereby to identify specific locus combinations of interest for further investigation and replication. Some new features were added to MDR through the extended MDR (EMDR) (Mei et al., in press). Basically, EMDR uses the same algorithm as the core MDR program, a data reduction program that tests for interactions (Ritchie et al. 2001; Hahn et al. 2003). EMDR contains several new features. Briefly, these are (1) allowance of missing data in individuals with partial genotype data, (2) use of a χ^2 statistic in addition to the prediction error as a test statistic, and (3) introduction and implementation of a nonfixed permutation test to assess the statistical significance of models identified by EMDR. This nonfixed permutation generates an empirical *P* value for a particular *n*-locus model, with consideration of all combinations of *n* loci. For example, for a particular 2-locus combination, the nonfixed permutation test accounts for the search of all possible 2-locus models to decide whether the best model

Table 5

Best Gene-Gene Effect Models Identified by EMDR for GABAR Subunit Genes on Chromosome 15

Gene and Marker	Marker Number	Location	Best Model	Nonfixed *P* Value[a]	
				χ^2	Misclassification
GABRB3:					
RS2081648	1	11.07	10	***.219***[b]	.706
RS1426217	2	11.08	5, 10	.494	.56
RS754185	3	11.23	4, 10, 13	.843	.85
HCV8865209	4	11.33	4, 5, 10, 13	.623	.875
RS2059574	5	11.54			
GABRA5:					
HCV42974	6	12.06			
RS140681	7	12.12			
RS140683	8	12.14			
GABRG3:					
HCV2078506	9	12.38			
RS208129	10	12.81			
RS897173	11	12.94			
HCV428306	12	14.46			
RS140679	13	14.66			

[a] Empirical *P* value derived from nonfixed permutation test by use of χ^2 or the misclassification rate as the test statistic in EMDR.

[b] The locus with the lowest *P* value (in bold italics) was selected as the one for the final cross-chromosome model.

is significant. An empirical *P* value <.05 was regarded as statistically significant and is inherently adjusted for multiple testing. In this study, a cross-validation option was not used.

For case-control pairs used in EMDR, the proband (or the most completely genotyped affected child) from each multiplex and triad family was selected ($n = 470$), and the untransmitted alleles were generated using parental genotypes as a control. Given the sample size of 470 case-control pairs in this study, we did not test for interactions that were >4-way interactions (Mei et al., in press).

Independent markers (tagging SNPs) were used in four chromosome-by-chromosome models and were selected using the confidence-interval function in Haploview (Barrett et al. 2005). Meanwhile, to retain adequate power to detect a gene-gene effect, the markers with the smallest *P* values from the four by-chromosome models were selected to build the final cross-chromosome model. The reason for the selection is that the permutation test inherently adjusts for multiple comparisons, and true effects can be overwhelmed when many markers are considered. Therefore, to maintain reasonable power, we judiciously chose a relatively small subset of markers for the MDR analysis. Our approach was staged so that each chromosome was examined and the markers having the smallest *P* values within each chromosome were selected for the overall analysis.

The significant best models identified by EMDR can only suggest a gene-gene effect, rather than a certain interaction. This holds true especially if a particular locus in a significant *n*-locus model also presents a significant main effect as the best 1-locus model. In this case, the identified gene-gene effect may be driven by the main effect from the locus, rather than by a true interaction. To verify the interaction between genes in the identified model, conditional logistic regression (by use of COXREG in SPSS, version 11.5 for Windows) was performed. To test for interaction, all variables (i.e., markers in the identified model) and their interaction terms were forced into the model. The genotypes of the markers were recoded in the logistic regression analysis. Genotypes with a case:control ratio >1 were collapsed and were recoded as the high-risk group, and those with a ratio <1 were recoded as the low-risk group. This matched the dimensionality-reduction strategy applied in EMDR, enabling consistent interpretation of the results between EMDR and the logistic regression analysis. In this study, GG was coded as a high-risk group for marker *RS1912960,* and GG and TT were coded as high-risk groups for *RS2351299*. Finally, multilocus geno-PDT and association in the presence of linkage (APL) analysis (Martin et al. 2003*b*) were used to validate the gene-gene interaction from the logistic regression.

Results

No significant deviation from HWE was found in unaffected whites for all SNPs. SNP *RS1426217* (in *GABRB3*) on chromosome 15 presented evidence of deviation from HWE in the affected individuals ($P = .019$). PDT showed that *RS1912960* (in *GABRA4*) on

Table 6

Best Gene-Gene Effect Models Identified by EMDR for All Known Autosomal GABAR Subunit Genes

Marker	Gene	Location	Chromosome	Marker Number	SNP Number	Best Model	Nonfixed *P* Value[a]	
							χ^2	Misclassification
RS1912960	*GABRA4*	65.857	4	9	1	1	.035	.02
RS2351299	*GABRB1*	65.916	4	13	2	1, 2	.002	.001
RS12187676	*GABRB2*	165.246	5	6	3	5, 6, 7	.009	.008
RS407206	*GABRR1*	94.935	6	2	4			
RS282117	*GABRR2*	95.171	6	8	5			
RS2148174	*GABRR2*	95.238	6	10	6			
RS208129	*GABRG3*	12.813	15	10	7			

[a] Empirical *P* value derived from nonfixed permutation test by use of χ^2 or the misclassification rate as the test statistic in EMDR.

chromosome 4 had a preferential transmission of the common G allele to the affected offspring ($P = .012$) (table 1). In addition, FBAT identified a significant association at *HCV9866022* (in *GABRR2*) on chromosome 6 ($P = .04$), where the PDT results suggested a similar trend ($P = .064$) (the entire FBAT data are not shown; results were similar to those of PDT). The geno-PDT displayed positive genotype association on chromosome 4 for homozygous common genotypes TT, GG, and GG at *HCV8262334* (in *GABRA2*), *RS1912960* (in *GABRA4*), and *RS2280073* (in *GABRA4*), respectively, and, on chromosome 5, for heterozygous genotypes CT and CG in *RS2617503* and *RS12187676* (both in *GABRB2*), respectively (global *P* values given in table 1). SNPs on the same chromosome did not show LD with each other (LD data not shown).

Haplotype analysis was performed using tagging SNPs within each gene, and it confirmed significant association with autism for specific haplotypes within *GABRA2* ($P = .027$), *GABRA4* ($P = .025$), and *GABRR2* ($P = .028$). However, the global *P* value was not significant ($P > .05$) for any of the genes tested.

Table 7

Results for Multilocus geno-PDT for Two Loci on Chromosome 4

Genotype[a]		*P* Value[b]
RS1912960	*RS2351299*	
1,1	1,1	.015
1,1	1,2	.330
1,1	2,2	.096
1,2	1,1	.061
1,2	1,2	.635
1,2	2,2	.835
2,2	1,1	***.001***
2,2	1,2	.046
2,2	2,2	.386

[a] For *RS1912960*, 1 = C; 2 = G (common allele); for *RS2351299*, 1 = G (common allele); 2 = T.

[b] *P* value for each genotype combination. The global *P* value (after adjustment for all possible genotype combinations) is .0007.

To test for a gene-gene effect, EMDR was run for by-chromosome and cross-chromosome models. Of all of the by-chromosome models tested (tables 2–6), two significant models were found on chromosome 4. There is a 2-locus model involving *RS1912960* in *GABRA4* and *RS2351299* in *GABRB1* ($P = .002$) and a 3-locus model involving *RS2350439* in *GABRG1*, *RS1912960* in *GABRA4*, and *RS3114084* in *GABRB1* ($P = .03$), which suggests a potential gene-gene interaction among *GABRG1*, *GABRA4*, and *GABRB1* (table 2). (The original MDR under the 10-fold cross-validation option was run, and it confirmed a potential gene-gene effect in a 2-locus model [prediction error = 43%; $P = .023$].) In the cross-chromosome model (table 6), EMDR identified the same best 1-locus and 2-locus ($P = .001$) models as in the by-chromosome model for chromosome 4 and confirmed the main effect at *RS1912960* (in *GABRA4*; $P = .02$). Another 3-locus model (*RS282117* and *RS2148174* in *GABRR2* and *RS208129* in *GABRG3*; $P = .008$) was also identified, suggesting a potential gene-gene interaction across chromosomes, between *GABRR2* (chromosome 6) and *GABRG3* (chromosome 15).

To evaluate whether the joint effects identified by the EMDR are the result of interacting genes, conditional logistic regression was conducted; the results supported a significant 2-locus gene-gene interaction between *GABRA4* and *GABRB1* (odds ratio [OR] of 2.9 for interaction term, high risk vs. low risk; $P = .002$) but did not detect an interaction in the cross-chromosome 3-locus or chromosome 4 3-locus model.

Consistent with the interaction term in the logistic regression described above (high risk [GG] and high risk [GG+TT] combination), multilocus geno-PDT (table 7) supported a positive cross-marker genotype association with disease between two common variant genotypes at *RS1912960* (GG) and *RS2351299* (GG). The APL analysis confirmed a positive association of the G allele

at *RS1912960* ($P = .031$) and also presented a positive haplotype association with disease for a haplotype with two common variants (G-G) (*RS1912960* and *RS2351299*; $P = .014$; global $P = .014$), which indirectly supported the genotype association shown by EMDR.

Discussion

This is, to our knowledge, the first comprehensive investigation of the allelic, genotypic, and haplotypic association together with an investigation of potential gene-gene interactions of all known autosomal GABAR subunit genes with autism. These novel findings indicate that *GABRA4* is involved in the etiology of autism, both independently and through interaction with *GABRA1*. These data support the hypothesis that complex interactions account for autism risk and present some of the first evidence for it.

In the present study, several approaches were used to control for false-positive results and thus to protect against incorrect conclusions regarding the etiology of the disease. First, we included only autism-affected white families in the analysis, to avoid biased results due to population stratification. Second, all GABA genes selected have a substantial a priori probability of involvement in autism (Sullivan et al. 2001). Finally, we used a multianalytic approach, as described elsewhere (Ashley-Koch et al. 2004), to interpret our findings. This approach looked for the convergence of results across several methods, rather than relying on results from a single analytic tool. Specifically, several approaches were applied to validate the interaction identified by EMDR, including conditional logistic regression.

To evaluate multilocus effects in a comprehensive way, the results from allelic, genotypic, and haplotypic analyses were integrated for a best estimate. We also used an extended version of MDR (Mei et al., in press), in which several modifications were made to MDR, including allowance for missing data, improved estimation of test statistic distribution, and more-accurate adjustment of multiple testing. These new features in EMDR have been validated elsewhere (Mei et al., in press). In this study, we chose to use the no-cross validation option and to omit the 10-fold cross-validation in each run. This option has shown lower false-positive and false-negative rates than the original MDR (Mei et al., in press).

We previously reported linkage and weak association for SNPs in the cluster GABAR region on chromosome 15q in our autism data set (Bass et al. 2000; Martin et al. 2000*a*; Menold et al. 2001; Shao et al. 2003). One possible explanation for this finding would be that there are multiple disease variants for autism risk in this region and that any one variant is only weakly associated with an individual haplotype. Similarly, the present study did not find association for a single locus in this region. However, *RS1426217,* which is located in intron 6 of *GABAB3,* significantly deviated from HWE only in affected individuals. This does not invalidate the association analysis, since both PDT and FBAT do not require HWE. It has been suggested elsewhere that absence of HWE may be an indication of the presence of association of a susceptibility allele that is in LD with the tested SNP (Nielsen et al. 1998). Thus, this finding might suggest that *RS1426217* is in LD with a nearby disease allele. Extending the analysis to chromosome 15 GABAR genes (*GABRB3, GABRA5,* and *GABRG3*), we applied a similar genetic analysis paradigm (Ashley-Koch et al. 2004) to look for interactions among these three genes to determine whether these interactions contribute to risk, but no multilocus effects were detected. In a cross-chromosome model, however, we did find a joint effect between *GABRR2* (chromosome 6) and *GABRG3* (chromosome 15), although conditional logistic regression failed to confirm this interaction. On the basis of the FBAT and HBAT results for *GABRR2,* this joint effect is most likely driven by the effect from *GABRR2* only. Even so, the finding merits further investigation in a larger and/or independent sample.

The most promising finding in this study was the significant allelic and genotypic association that was found at *RS1912960* (in *GABRA4*), both from the common variant G and the common genotype GG. Also, the HBAT identified a significant haplotype within *GABRA4,* although the global *P* value showed only marginal significance ($P = .06$). Moreover, the association remained significant even after adjustment for multiple testing in EMDR. The program generates 1,000 simulated data sets by permuting the status of cases and controls to obtain an empirical *P* value for the marker while testing the significance for the 1-locus best model. *RS1912960* remained the best 1-locus model in both the by-chromosome and the cross-chromosome models. The empirical *P* value for this marker was .038 for the by-chromosome model and .020 for the cross-chromosome model. Thus, this significant association appeared to be consistent across all analyses, which strongly suggests that *GABRA4* is involved in the etiology of autism.

Although no strong linkage or significant association at *GABRA4* had previously been reported, two genome screens reported suggestive linkage at 4p (International Molecular Genetic Study of Autism Consortium 1998; Auranen et al. 2000). Two chromosomal studies indicated abnormality in the 4p12 region, which is at the same location as *GABRA4*: a British group reported a 46,XX, dup (4) p12-p13 chromosome abnormality in an 18-year-old female with autism (Sabaratnam et al. 2000), and a Canadian group reported a paracentric inversion of the short arm of chromosome

4 (46,XY, inv [4] p12-p15.3) in two brothers with autism (Choufani et al. 2003). Given the convergence of all available results, we believe the positive association at *RS1912960* (in *GABRA4*) reflects a true association with autism.

Relatively little is known about the biological function(s) of the GABARA4 subunit. Gene expression is known to be highly variable, depending on brain region, neuronal activity, and development, which suggests complex regulation and involvement in multiple brain activities and functions. Levels of α4 mRNA are found in the hippocampus, the dentate gyrus, the thalamus, the nucleus accumbens, the cerebellum, the outer layers of the cortex, and other regions, and they peak during development. Unlike most $GABA_A$ receptor complexes, those containing α4 are not sensitive to modulation by diazepam. It has been suggested that the α4 subunit may be involved in neuronal hyperexcitability. The promoter for α4 has multiple transcription initiation sites, and alternate splicing in mouse brain has been observed (Ma et al. 2004).

We also found a potential interaction between *GABRA4* and another clustering GABA gene, *GABRB1* (OR = 2.9 for interaction term). We identified this potential gene-gene effect model in EMDR from both the by-chromosome and the cross-chromosome models. In addition, the interaction was further confirmed by conditional logistic regression, in which two common GG-GG variant combinations substantially increased autism risk. This finding is also consistent with the results from multilocus geno-PDT (GG-GG) and APL haplotype analysis (G-G). Again, the accumulation of findings across all analyses leads us to conclude that *GABRB1* may be involved through interaction with *GABRA4*.

In conclusion, this study suggests that the *GABRA4* gene is involved in the etiology of autism and substantially increases autism risk through interaction with the *GABRB1* gene. Furthermore, these findings support the hypothesis that the etiology of autism is complex and that complex interactions may contribute to autism risk.

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Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for autistic disorder)

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