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Variations in *GABRA2,* Encoding the α2 Subunit of the $GABA_A$ Receptor, Are Associated with Alcohol Dependence and with Brain Oscillations

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Alcoholism is a complex disease with both genetic and environmental risk factors. To identify genes that affect the risk for alcoholism, we systematically ascertained and carefully assessed individuals in families with multiple alcoholics. Linkage and association analyses suggested that a region of chromosome 4p contained genes affecting a quantitative endophenotype, brain oscillations in the beta frequency range (13–28 Hz), and the risk for alcoholism. To identify the individual genes that affect these phenotypes, we performed linkage disequilibrium analyses of 69 single-nucleotide polymorphism (SNPs) within a cluster of four $GABA_A$ receptor genes, *GABRG1, GABRA2, GABRA4,* and *GABRB1,* at the center of the linked region. $GABA_A$ receptors mediate important effects of alcohol and also modulate beta frequencies. Thirty-one SNPs in *GABRA2,* but only 1 of the 20 SNPs in the flanking genes, showed significant association with alcoholism. Twenty-five of the *GABRA2* SNPs, but only one of the SNPs in the flanking genes, were associated with the brain oscillations in the beta frequency. The region of strongest association with alcohol dependence extended from intron 3 past the 3′ end of *GABRA2*; all 43 of the consecutive three-SNP haplotypes in this region of *GABRA2* were highly significant. A three-SNP haplotype was associated with alcoholism, with $P = .000000022$. No coding differences were found between the high-risk and low-risk haplotypes, suggesting that the effect is mediated through gene regulation. The very strong association of *GABRA2* with both alcohol dependence and the beta frequency of the electroencephalogram, combined with biological evidence for a role of this gene in both phenotypes, suggest that *GABRA2* might influence susceptibility to alcohol dependence by modulating the level of neural excitation.

Introduction

Alcohol dependence (alcoholism [MIM 103780]) is a common, complex disease with both genetic and environmental risk factors. Both twin studies and adoption studies demonstrate a substantial heritable component to the risk for alcoholism (Goodwin 1979; Pickens et al. 1991; Kendler et al. 1994; Heath et al. 1997). The increased risk for alcoholism among first-degree relatives of alcohol-dependent individuals is in the range of three- to eightfold (Reich et al. 1998). However, there is no simple pattern of inheritance, suggesting that multiple genes and their interaction with each other and with the environment are involved. Therefore, identification of genes that affect the risk for alcoholism has been a difficult endeavor.

To identify genes that affect the risk for alcoholism, the Collaborative Study on the Genetics of Alcoholism (COGA) systematically ascertained and studied a large collection of families containing at least three alcoholic members (Begleiter et al. 1995; Foroud et al. 2000; Edenberg 2002). Our strategy involved focusing genetic efforts on families containing at least three alcoholic members. These families are likely to have a greater genetic contribution to susceptibility and, thus, may provide increased power to detect genes contributing to this complex phenotype. A whole-genome survey using sibling-pair linkage analysis identified several chromosomal regions for which there was evidence of a gene or genes affecting alcohol dependence (Reich et al. 1998; Foroud et al. 2000; Edenberg 2002). There was evidence for in-

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† Deceased after submission of this article. We dedicate this article to Ted Reich's memory.

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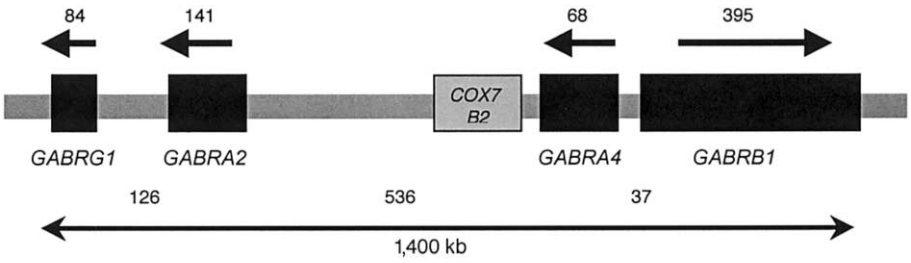

Figure 1 $GABA_A$ receptor gene cluster on chromosome 4, based on NCBI human genome build 33. Distances are in kilobases.

creased allele sharing at a microsatellite marker in the *GABRB1* locus on chromosome 4p (Reich et al. 1998). Studies of a different population also provided evidence for linkage of alcohol dependence with a marker very near the *GABRB1* gene (Long et al. 1998). Linkage disequilibrium (LD) analyses of the COGA families supported the association of alcoholism with a microsatellite marker in *GABRB1,* although the evidence was modest (Song et al. 2003).

The definition of alcohol dependence includes serious dysfunction in different domains, including tolerance to the effects of ethanol, a withdrawal syndrome during abstinence, craving for ethanol, and persistent drinking in the face of adverse consequences (American Psychiatric Association 1987, 1994; World Health Organization 1993). Given the heterogeneity inherent in the diagnosis of alcohol dependence, we collected extensive phenotypic data on the available individuals in these families, so that complementary analyses of alcoholism-related endophenotypes could be employed in the search for genes affecting alcoholism (Edenberg 2002).

Biological endophenotypes related to neurological function may help identify predisposing factors that increase the vulnerability to alcohol dependence (Gottesman and Gould 2003). We focused on brain oscillations, as measured by electroencephalography (EEG), as key endophenotypes (Porjesz et al. 2002*b*). Alcoholics differed from controls by having increased power in the beta frequency band (13–28 Hz) of the EEG (Costa and Bauer 1997; Rangaswamy et al. 2002). The offspring of male alcoholics also showed this difference (Bauer and Hesselbrock 1993; Rangaswamy et al. 2004). These data suggested that the EEG power in the beta frequency band provided a heritable, relevant endophenotype for analysis. Another advantage of this quantitative endophenotype is that we could use data from all individuals in the family, whether or not they met the criteria for alcohol dependence. We found particularly strong linkage for an EEG-β phenotype (see the "Methods" section) in a region of chromosome 4p around a microsatellite marker in *GABRB1,* with a LOD score of 5.0 (Porjesz et al. 2002*a*). The evidence for this localization increased to LOD 6.5 when association was also analyzed (Porjesz et al. 2002*a*). A nonparametric linkage analysis (Ghosh et al. 2003) of EEG-β also showed linkage to this region of chromosome 4 ($P < .000001$).

The very strong evidence that this region of chromosome 4p contains genes affecting the beta frequency of the EEG, combined with the evidence for an effect upon the risk for alcoholism, led us to pursue the identification of individual genes that affect these phenotypes. We used public databases to identify genes predicted to lie within the 16-cM region of chromosome 4p that represented a 1-LOD interval around the peak of the EEG linkage (Porjesz et al. 2002*a*). There is a cluster of genes encoding subunits of the $GABA_A$ receptor centered within this region: *GABRG1, GABRA2, GABRA4,* and *GABRB1* (fig. 1). γ-Amino butyric acid (GABA) is the principal inhibitory neurotransmitter in the vertebrate brain (Barnard et al. 1998). $GABA_A$ receptors are pentameric assemblies of subunits; 19 mammalian subunits are known, which are classified into α, β, γ, δ, ε, π, and ρ types (Barnard et al. 1998). Most GABA receptors contain α, β, and γ subunits (Barnard et al. 1998; Sieghart et al. 1999). Binding of GABA to ionotropic $GABA_A$ receptors opens an integral chloride-ion channel that alters the membrane potential of neurons. $GABA_A$ receptors mediate fast synaptic inhibition, and beta oscillations involve $GABA_A$-receptor action (Haenschel et al. 2000), making the genes encoding receptor subunits strong candidates for the genes within this region that affect brain oscillations.

Important effects of alcohol, including disruption of motor coordination, anxiolysis, sedation, symptoms related to withdrawal, and ethanol preference, are mediated by GABA transmission (Buck 1996; Grobin et al. 1998; Korpi et al. 1998). $GABA_A$ agonists increase ethanol intake in rats, whereas $GABA_A$ antagonists decrease intake (Boyle et al. 1993; Tomkins and Fletcher 1996; Nowak et al. 1998). The effects vary in different brain regions, perhaps because of the presence of receptors composed of different groups of subunits (Barnard et al. 1998; Grobin et al. 1998). A variation in the *Gabrg2* gene in mice correlates with the severity of acute alcohol withdrawal (Buck and Hood 1998) and with ethanol-induced motor incoordination, hypothermia,

and ethanol-conditioned taste aversion (Hood and Buck 2000). These lines of biological evidence make GABA receptor genes excellent candidates to contribute to differences in risk for alcoholism.

Therefore, the four genes in this region of chromosome 4p that encode subunits of the $GABA_A$ receptor were our highest-priority candidates for study. We report detailed analysis of 69 SNPs across the 1.4-Mb cluster of four $GABA_A$ receptor subunit genes. We analyzed LD between the markers and determined that the effects of individual genes could be discriminated. We found very strong evidence that one of these genes, *GABRA2,* is strongly associated with both alcohol dependence and brain oscillations.

Material and Methods

Subjects

A large collection of families containing multiple alcoholics was systematically ascertained by the COGA through probands in treatment for alcohol dependence (Reich et al. 1998; Foroud et al. 2000). For acceptance into the genetic part of this study, at least two other first-degree relatives of the proband also had to be alcohol dependent, as assessed by direct interview. Informed consent was obtained after the nature and possible consequences of the studies were explained. Diagnostic data were obtained by direct interview using the Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz et al. 1994).

Electrophysiology

EEG recordings were obtained with noninvasive scalp electrodes in awake individuals with their eyes closed. The filtered artifact-free data were transformed into horizontal bipolar derivations (Porjesz et al. 2002*a*). Absolute power over the range between 3 and 28 Hz was subdivided into theta (3.0–7.0 Hz), alpha 1 (7.5–9.0 Hz), alpha 2 (9.5–12.0 Hz), beta 1 (12.5–16.0 Hz), beta 2 (16.5–20.0 Hz), and beta 3 (20.5–28.0 Hz) frequency bands. A singular value decomposition procedure (Wang et al. 2000) was utilized to obtain phenotypic data for each of the six EEG bands. Since the beta 2 phenotype yielded highly significant linkage and combined linkage/LD (Almasy et al. 1999) with the *GABRB1* microsatellite marker on chromosome 4 (Porjesz et al. 2002*a*), we assessed differences in this EEG phenotype (hereafter referred to as "EEG-β") by SNP genotype through use of a measured genotype test (Boerwinkle et al. 1986) implemented in SOLAR (Almasy and Blangero 1998). An additive model was assumed, with heterozygotes having a genotypic mean halfway between that of the two homozygotes, and differences in genotype-specific trait means were tested using a likelihood-ratio comparison.

SNP Genotyping and Analysis

SNPs were identified from public databases, including dbSNP, the National Center for Biotechnology Information (NCBI) Reference Sequence project (for human genome contigs), and LocusLink, as well as by DNA sequencing (below). Contigs containing the $GABA_A$ receptor genes in the chromosome 4 cluster were downloaded from NCBI into a database we constructed to facilitate choosing SNPs in and near the genes. Positions shown are based on the NCBI human genome build 33 and dbSNP build 116 (table 1; fig. 1). Rather than examine a limited number of coding SNPs, which are usually of low heterozygosity, we genotyped SNPs of high allele frequency across *GABRG1, GABRA2, GABRA4,* and *GABRB1*. When our initial data strongly suggested that variations in *GABRA2* were associated with both alcohol dependence and EEG-β, we sequenced exons of this gene to find additional variants (below), genotyped several in the full sample, and genotyped additional SNPs in *GABRA2*.

Assays were designed using SpectroDESIGNER software (Sequenom) and performed using a modified single-nucleotide extension reaction with allele discrimination by mass spectrometry (Sequenom MassArray system) (Jurinke et al. 2001). Genotypes were tested for Mendelian inheritance, and inconsistent genotypes (an average of 14 of 3,560; 23 SNPs had $\leq$10 inconsistencies) were discarded.

LD between markers was analyzed using the program GOLD (Abecasis and Cookson 2000). To examine association between the SNPs and the phenotype of alcohol dependence, we used the pedigree disequilibrium test (PDT) (Martin et al. 2000), which uses information from the entire pedigree rather than just the affected subject and his/her two parents and thus takes advantage of the extended families that we are studying. Family-based association studies avoid the problems of false positive results arising from population stratification, which can occur in population-based association approaches (Spielman and Ewens 1996). We analyzed two haplotypes constructed from three SNPs each: one based on rs279871, rs279826, and rs279836 (chosen on the basis of their significant association with alcohol dependence; table 1), and one based on rs279871, rs279845, and rs279836 (chosen because of their more significant association with EEG-β; tables 1 and 2). We then systematically examined all consecutive three-SNP haplotypes of *GABRA2* (table 3).

DNA Sequencing

To examine whether there were coding or splice-site polymorphisms within the *GABRA2* gene, the entire coding region of the *GABRA2* gene was sequenced in DNAs from 48 individuals, 25 of whom were homozygous for a strongly associated haplotype

Table 1

Association of SNPs with DSM-IV Alcohol Dependence and EEG

Marker[a]	Gene	Function	Number[b]	Position[c]	Alcohol Dependence *P* Value[d]	EEG-β *P* Value[e]
rs1497570	*GABRG1*	IVS8		45896957	.65	.68
rs1948609	*GABRG1*	IVS5		45912695	.88	.86
rs1391175	*GABRG1*	IVS1		45951087	.050*	.41
rs2221020	*GABRG1*	IVS1		45953488	.068	.98
rs1391168	*GABRG1*	IVS1		45965082	.45	.85
rs904154	*GABRG1*	5′	1	45976294	.19	.18
rs490434	*GABRA2*	3′	2	46043202	.0052*	.64
rs576666	*GABRA2*	3′	3	46052300	.095	.067
rs531460	*GABRA2*	3′	4	46060193	.022*	.024*
rs561779	*GABRA2*	3′	5	46088701	.048*	.044*
rs495818	*GABRA2*	3′	6	46097821	.022*	.034*
rs497068	*GABRA2*	3′		46100600	.0069*	.26
rs572227	*GABRA2*	3′		46101316	.038*	.019*
rs573400	*GABRA2*	3′UTR		46101989	.062	.27
rs541418	*GABRA2*	IVS9		46103139	.020*	.10
rs481311	*GABRA2*	IVS9		46104305	.076	.17
rs507788	*GABRA2*	IVS9	7	46107381	.031*	.068*
rs532780	*GABRA2*	IVS9		46111289	.079	.016*
rs548583	*GABRA2*	IVS9		46113267	.012*	.028*
ss15649713	*GABRA2*	IVS9		46113787	.103	.56
rs496650	*GABRA2*	IVS8	8	46114308	.054	.75
rs540363	*GABRA2*	IVS8	9	46124169	.044*	.49
rs526752	*GABRA2*	IVS8		46126552	.12	.07
rs530329	*GABRA2*	IVS8		46131042	.034*	.048*
rs483160	*GABRA2*	IVS8	10	46136998	.15	.036*
rs279871[f]	*GABRA2*	IVS7	11	46155656	.0004*	.049*
rs279867	*GABRA2*	IVS6		46158226	.24	.05*
rs279866	*GABRA2*	IVS6		46159687	.029*	.037*
rs279863	*GABRA2*	IVS5		46162945	.017*	.011*
rs279861	*GABRA2*	IVS5		46163248	.037*	.045*
rs279858	*GABRA2*	Exon 5	12	46164516	.0087*	.22
rs175931	*GABRA2*	IVS4		46166246	.10	.071*
rs279843	*GABRA2*	IVS4	13	46175127	.049*	.30
rs279845[f]	*GABRA2*	IVS4	14	46179646	.013*	.011*
rs279846	*GABRA2*	IVS4		46179809	.017*	.012*
rs183961	*GABRA2*	IVS4		46180951	.038*	.014*
rs1440130	*GABRA2*	IVS4		46183176	.013*	.017*
rs279826	*GABRA2*	IVS4	15	46184132	.0008*	.25
ss15649712	*GABRA2*	IVS4		46184417	.014*	.70
rs279827	*GABRA2*	IVS3		46184625	.0068*	.016*
rs279828	*GABRA2*	IVS3		46184733	.0086*	.02*
rs279834	*GABRA2*	IVS3		46188222	.015*	.027*
rs279836[f]	*GABRA2*	IVS3	16	46188993	.0071*	.0066*
rs279837	*GABRA2*	IVS3		46189246	.035*	.064
rs279841	*GABRA2*	IVS3		46190686	.038*	.018*
rs189957	*GABRA2*	IVS3	17	46196602	.053*	.27
rs1442059	*GABRA2*	IVS3	18	46206875	.034*	.018*
rs1442061	*GABRA2*	IVS3		46221143	.37	.24
rs1442062	*GABRA2*	IVS3	19	46226999	.22	.13
ss15649711	*GABRA2*	IVS3	20	46237967	.76	.57
ss15649710	*GABRA2*	IVS 1		46240788	.91	.97
rs3756007	*GABRA2*	IVS 1		46240987	.99	.98
rs894269	*GABRA2*	5′	21	46243535	.097	.84
rs2165607	*GABRA2*	5′		46250144	.44	.67
rs1545234	*GABRA2*	5′	22	46254336	.41	.62

(continued)

Table 1 (continued)

Marker[a]	Gene	Function	Number[b]	Position[c]	Alcohol Dependence *P* Value[d]	EEG-β *P* Value[e]
rs2036943	Intergenic	5′	23	46726482	.85	.030*
rs2055943	*GABRA4*	IVS7		46817202	.72	.70
rs1512135	*GABRA4*	IVS6		46823811	.63	.38
rs1877400	*GABRA4*	IVS5		46827224	.90	1.00
rs2280072	*GABRA4*	IVS1		46845093	.53	.79
rs2280071	*GABRA4*	5′UTR		46845289	.98	.96
rs2055940	*GABRA4*	5′UTR		46847836	.36	.29
rs2119780	*GABRB1*	IVS4		47014133	.33	1.00
rs989808	*GABRB1*	IVS4		47016704	.77	.50
rs1372496	*GABRB1*	IVS4		47057731	.99	.53
rs1372497	*GABRB1*	IVS4		47072261	.53	.81
rs6284	*GABRB1*	IVS5		47172142	.98	.54
rs2070922	*GABRB1*	IVS7		47255971	.22	.61
rs6289	*GABRB1*	IVS8		47258632	.63	.66

NOTE.—Significant *P* values are indicated with an asterisk (*).

[a] Markers are shown as rs numbers (or ss numbers, for those we submitted that are not yet included) from the dbSNP database.

[b] Refers to the numbering in figure 2.

[c] Position is in nucleotides from chromosome 4pter, as estimated in the dbSNP database (build 116) or by blasting against the NCBI Human Genome assembly (build 33).

[d] *P* value for alcohol dependence, based on the average PDT (Martin et al. 2000).

[e] *P* value for EEG-β, based on a measured genotype test (Boerwinkle et al. 1986) implemented in SOLAR (Almasy and Blangero 1998).

[f] Marker used for the haplotype analysis in table 2.

($P = .000024$) consisting of rs279871, rs279826, and rs279836; 21 of whom were homozygous for the most common nonassociated haplotype; and 2 of whom were homozygous for uncommon haplotypes. The coding regions plus at least 60 bp of each flanking intronic sequence were amplified by PCR and sequenced in both directions, using the ABI PRISM 3100 Genetic Analyzer capillary DNA sequencer with Big Dye chemistry (Applied Biosystems). Additional SNPs were found, several of which were genotyped in the full sample; these have been submitted to dbSNP (the ss numbers are given in table 1).

Results

To test whether individual gene(s) within the region of linkage on chromosome 4p affect either the neurophysiological or alcohol-dependence phenotype, we initially genotyped and analyzed 20 SNPs across the 1.4-Mb cluster that contains *GABRG1, GABRA2, GABRA4,* and *GABRB1* (fig. 1). LD between markers within each gene was high (fig. 2). LD between genes was considerably lower than that within a gene; in particular, LD drops at both ends of *GABRA2*. This is consistent with other data on LD across the human genome (Daly et al. 2001; Goldstein 2001; Reich et al. 2001; Gabriel et al. 2002). Thus, our analysis of the association of alcoholism with the $GABA_A$ receptor genes can differentiate among the genes, even though they are physically close together.

We used a family-based association method, the PDT (Martin et al. 2000), to examine association between the SNPs and the phenotype of alcohol dependence, as defined by DSM-IV criteria (American Psychiatric Association 1994). Our initial analyses showed that eight of nine SNPs in the *GABRA2* gene were significantly associated with alcohol dependence. In contrast, SNPs in the three other $GABA_A$ receptor genes in this cluster, including genes on either side of *GABRA2,* did not show a pattern of association with alcohol dependence. Therefore, additional SNPs in *GABRA2* were genotyped and continued to show association with alcohol dependence and with EEG-β (table 1). Thirty-one SNPs within or closely flanking *GABRA2* were associated with alcohol dependence, at *P* values $\leqslant .05$ (table 1). The region associated with alcohol dependence spanned 164 kb, extending from intron 3 to 58 kb past the 3′ end of the gene.

Twenty-five of the SNPs within *GABRA2* and one near it were associated with EEG-β (table 1), the endophenotype previously analyzed in the whole-genome survey (Porjesz et al. 2002*a*). SNPs in the other flanking $GABA_A$ receptor genes were not associated with this phenotype.

To further analyze the association of the *GABRA2*

Table 2

Association of Haplotypes with Alcohol Dependence

Haplotype[a]	Frequency	% of All Haplotypes	Parental Contribution		No. of Discordant Sibs		*P* Value[b]
			No. of Transmitted Alleles	No. of Nontransmitted Alleles	Affected	Unaffected	
1	287	5.5	76	69	67	61	.66
2	226	4.3	57	61	44	39	.94
3	74	1.4	11	19	8	17	.25
4	2,531	48.5	728	589	549	518	.000000022
5	1,724	33.1	454	437	354	395	.67
6	68	1.3	8	15	8	11	.70
7	54	1.0	9	17	3	5	.96
8	252	4.8	59	77	47	60	.17

[a] Haplotype derived from three markers (see footnote "f" in table 1); the high-risk haplotype (4) is rs279871 (A), rs279845 (T), rs279836 (A).

[b] *P* values are based on the average PDT (Martin et al. 2000), treating each haplotype as a marker in an eight-allele system.

gene with alcohol dependence, a set of three SNPs that showed association with both alcohol dependence and EEG-β was analyzed as a haplotype (table 2). The global PDT was significant ($P = .0001$). The higher-risk haplotype, consisting of the alleles that were overtransmitted in the individual SNP analyses, was associated with alcohol dependence at $P = .00000002$. We then systematically examined, by the global PDT test, all consecutive three-SNP haplotypes within *GABRA2* and found that 1 of the 5 haplotypes at the 5′ end of the gene and all 43 of the haplotypes starting within exon 3 and extending to the 3′ end of the gene were significantly associated with alcohol dependence (table 3). The median global significance of the 43 haplotypes from intron 3 to the end of the gene was .007; the most significant were $\leq .000001$. The high-risk haplotypes themselves had a median significance of .000098 (table 3).

We examined the prevalence of the higher-risk haplotype by sex. Among alcohol-dependent individuals, the same proportion of males (50%) and females (53%) have the higher-risk haplotype. Looking at the data in a different way, 46% of males who have the high-risk haplotype are affected, whereas only 25% of the females who have the high-risk haplotype are affected; this is reasonable, given the lower prevalence of alcoholism in females.

None of the SNPs tested affect the amino acid sequence of the encoded $\alpha 2$ subunit. To examine whether there were coding polymorphisms in LD with the tested SNPs, we sequenced DNA from 48 individuals, 25 of whom were homozygous for a strongly associated high-risk haplotype ($P = .000024$) consisting of rs279871, rs279826, and rs279836; 21 of whom were homozygous for the most common nonassociated haplotype; and 2 of whom were homozygous for uncommon haplotypes. No polymorphisms that altered the amino acids encoded by the *GABRA2* gene were found.

Discussion

Our results provide extremely strong evidence that variations in the *GABRA2* gene, and not in the flanking $GABA_A$ receptor genes, affect both brain oscillations in the beta frequency range (EEG-β) and alcohol dependence. We were led to examine genes in this region by strong linkage with EEG-β, demonstrated by two different methods of analysis: a variance-component method with additional evidence for association (Porjesz et al. 2002*a*) and a nonparametric analysis (Ghosh et al. 2003). The location and function of a set of $GABA_A$ receptor genes in the center of this linkage peak made them excellent candidate genes. Rather than examine a single SNP in each gene, we analyzed a total of 69 SNPs in four genes. Only one gene was consistently associated with alcohol dependence and with the EEG endophenotype: *GABRA2*.

Thirty-one SNPs in *GABRA2* were associated with alcohol dependence, and 25 were associated with EEG-β. The *P* value for association of a three-SNP haplotype with alcohol dependence, .000000022, surpasses the level of genomewide significance. The higher-risk haplotype is common (48.5%). Because so many haplotypes were associated (43 consecutive three-SNP haplotypes out of 47 tested and both selected haplotypes), we do not think there is any reasonable likelihood that the findings are artifacts of multiple testing. The significance of the high-risk haplotype remains after even the most conservative correction for multiple testing (Bonferroni, which assumes independence, an assumption clearly not true in this region of high LD, making the correction overly conservative). A Bonferroni correction would require $P < .001$, given that we examined 49 three-SNP haplotypes (47 systematically derived from three adjacent SNPs [table 3] and 2 selected from the individual SNPs with the greatest evidence of transmission distortion [table 2 and data not shown]). In fact, 8 of the 49 haplotype analyses surpassed that level of overall sig-

Table 3

Association between Consecutive Three-SNP Haplotypes in *GABRA2* and Alcohol Dependence

Marker	Overall Significance[a]	Significance of High-Risk Haplotype[b]
rs490434	...	...
rs576666	...	...
rs531460	.0066	.000034
rs561779	.0030	.000075
rs495818	.0064	.000077
rs497068	.0075	.00010
rs572227	.0037	.000035
rs573400	.0113	.00050
rs541418	.0088	.0011
rs481311	.0164	.00010
rs507788	.0127	.00020
rs532780	.0428	.00070
rs548583	.0028	.000039
ss15649713	.0258	.00050
rs496650	.0187	.0010
rs540363	.0427	.0010
rs526752	.0156	.00020
rs530329	.0060	.00020
rs483160	.0071	.000084
rs279871	.00010	.000003
rs279867	.000000	.000001
rs279866	.000003	.000001
rs279863	.00090	.000004
rs279861	.0052	.000050
rs279858	.0086	.00030
rs175931	.0030	.00010
rs279843	.0082	.000096
rs279845	.00080	.000013
rs279846	.0340	.0014
rs183961	.0042	.000051
rs1440130	.0018	.000015
rs279826	.0042	.000061
ss15649712	.0318	.0062
rs279827	.0240	.0068
rs279828	.0114	.0029
rs279834	.0141	.000041
rs279836	.0011	.000004
rs279837	.0025	.000019
rs279841	.0019	.000023
rs189957	.00080	.000027
rs1442059	.031	.00020
rs1442061	.022	.0030
rs1442062	.013	.00010
ss15649712	.026	.00040
ss15649710	.059	.0040
rs3756007	.35	.0275
rs894269	.15	.0026
rs2165607	.026	.0023
rs1545234	.24	.0865

NOTE.—Each set of three consecutive SNPs through the *GABRA2* gene was analyzed as an eight-allele marker, named by the third SNP in the set.

[a] *P* value based on the average PDT (Martin et al. 2000), treating each haplotype as a marker in an eight-allele system; the value is shown at the third of the three SNPs used to create each haplotype.

[b] Significance of the high-risk haplotype within each set of three consecutive SNPs.

nificance, and 23 of the high-risk haplotypes surpassed a significance level of .0001 (table 3).

Our strategy of genotyping multiple SNPs of high heterozygosity rather than focusing on coding SNPs (which are often of low heterozygosity) was driven by our hypothesis that the variations underlying complex genetic diseases predominantly affect gene regulation rather than the structure of the encoded protein. Gene regulation is the result of the combinatorial action of multiple transcription factors binding at multiple sites in and near a gene and therefore can be affected by multiple SNPs. We did not find any coding difference between the higher-risk and lower-risk haplotypes. The lack of coding SNPs in *GABRA2* shows that our strategy was advantageous; the alternative approach would not have allowed us to test the association. The association extends across a significant fraction of the *GABRA2* gene. It is perhaps surprising that it is strongest from intron 3 through the 3′ end of the gene rather than the 5′ region in which the promoter lies. Although effects of distal regulatory elements on transcriptional initiation are not ruled out, the data suggest that alternative splicing, mRNA stability, or other posttranscriptional mechanisms might be involved.

There are several lines of evidence suggesting biological links among *GABRA2*, alcoholism, and the EEG power spectral density in the beta range. $GABA_A$ re-

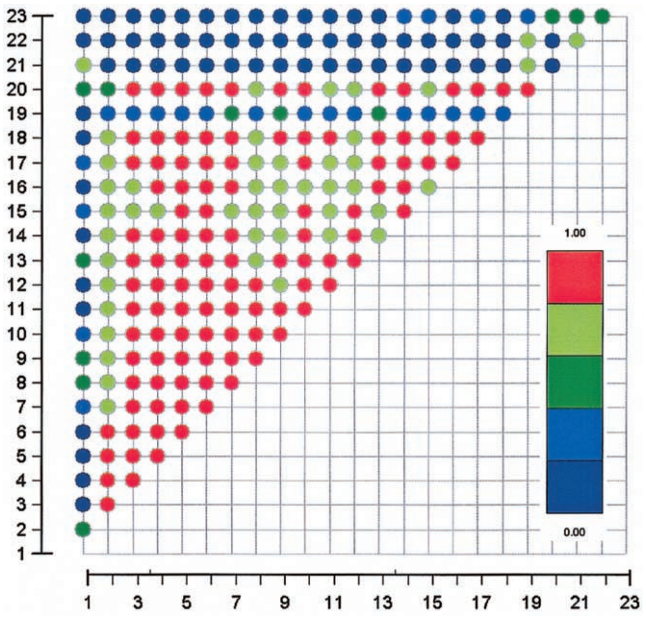

Figure 2 Pattern of LD within *GABRA2*. The numbers on the *X*- and *Y*-axes correspond to 21 markers selected to cover the *GABRA2* gene relatively evenly at an average spacing of 10.6 kb, plus two flanking markers; the markers are numbered in table 1. Each colored circle represents the LD between two markers, as measured by *D*′ (Abecasis and Cookson 2000). Higher values of *D*′ indicate higher LD.

ceptors have integral chloride channels that are opened by the binding of GABA, the major inhibitory neurotransmitter in the vertebrate brain (Barnard et al. 1998). The resulting chloride flux alters the membrane potential of neurons, inhibiting firing. $GABA_A$ receptors are sensitive to ethanol and are believed to mediate many of its effects, including anxiolysis, sedation, disruption of motor coordination, tolerance, and dependence (Grobin et al. 1998; Harris et al. 1998; Korpi et al. 1998; Buck and Finn 2001; Ueno et al. 2001). In rats, ethanol intake is increased by $GABA_A$ agonists and decreased by $GABA_A$ antagonists (Boyle et al. 1993; Tomkins and Fletcher 1996; Nowak et al. 1998), although the effects vary in different brain regions, perhaps because of differences in the subunit composition of the receptors (Barnard et al. 1998; Grobin et al. 1998). A variation in the *Gabrg2* gene in mice correlates with the severity of alcohol withdrawal, motor incoordination, ethanol-conditioned taste aversion, and hypothermia (Buck and Hood 1998; Hood and Buck 2000).

The $\alpha 2$ subunit of the $GABA_A$ receptor is a target of benzodiazepines (Harris et al. 1998; Low et al. 2000; Tobler et al. 2001), which are used in the treatment of alcohol withdrawal symptoms. Mice in which the $\alpha 2$ subunit was made insensitive to diazepam by introducing a point mutation into the *Gabra2* gene were insensitive to the anxiolytic effects of diazepam but retained sensitivity to its sedative and amnestic properties (Low et al. 2000); similar alteration to the *Gabra3* gene did not affect anxiolysis. This demonstrates that the $\alpha 2$ subunit is critical for anxiolysis.

Benzodiazepines strongly increase EEG beta power (Whittington et al. 1996; Traub et al. 1999), particularly in frontal regions (Traub et al. 1999). The $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits of the $GABA_A$ receptor mediate the effects of benzodiazepines on EEG spectral changes (Tobler et al. 2001). There is a significant increase in beta power in alcohol dependent subjects, particularly over frontocentral leads (Costa and Bauer 1997; Rangaswamy et al. 2002). This higher beta power was also found in offspring of male alcoholics (Bauer and Hesselbrock 1993; Bauer 2001; Rangaswamy et al. 2004). Bauer and Hesselbrock (1993) reported that enhanced high-frequency beta activity, originating from deep anterior regions of the frontal brain, was the best predictor of relapse in substance-dependent patients and may also be related to initial risk for dependence. They proposed that relapse, a key feature of alcoholism, was in part due to a deficit in function of the brain region that normally dampens impulsivity.

In addition to the separate evidence making GABA receptor genes good candidates for affecting both neurophysiological and diagnostic phenotypes, there are links between the phenotypes in these two domains. Begleiter and Porjesz (1999) hypothesized that neural disinhibition is involved in predisposition toward alcoholism. Oscillations in the beta (12.5–28 Hz) and gamma (28.5–50 Hz) frequencies are believed to represent an activated state of the neuronal network that generates them (Haenschel et al. 2000), so differences in these oscillations could affect the overall level of neural excitation and thereby the risk for alcoholism.

Our finding of very strong LD with alcohol dependence and with brain oscillations across the *GABRA2* gene, combined with the evidence for an effect of this gene on brain oscillations (Porjesz et al. 2002*a*) and anxiolysis (Low et al. 2000), draws these lines of evidence together. These data suggest that differences in the expression or function of $GABA_A$ receptors can modulate both the beta activity and neural inhibition. Our results also illustrate the power of studying endophenotypes to aid in the identification of genes affecting complex diseases: we were led to this region, in large part, by the very strong linkage to an electrophysiological endophenotype. The *GABRA2* gene proved to be associated both with the endophenotype and with alcoholism. The convergence of evidence from different analyses and phenotypes, along with the biological data on its function, provides strong evidence that *GABRA2* is a key gene affecting the risk for alcoholism.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for markers listed in tables 1 and 3, including new SNPs submitted: ss15649710, ss15649711, ss15649712, and ss15649713)

LocusLink, http://www.ncbi.nlm.nih.gov/LocusLink/

NCBI Reference Sequence, http://www.ncbi.nlm.nih.gov/RefSeq/

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

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