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GABRR1 and *GABRR2*, Encoding the GABA-A Receptor Subunits ρ1 and ρ2, Are Associated With Alcohol Dependence

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The genes encoding several GABA-A receptor subunits, including *GABRA2*, have been associated with alcoholism, suggesting that variations in gaba signaling contribute to risk. Therefore, as part of a comprehensive evaluation of the GABA receptor genes, we evaluated the potential association of *GABRR1* and *GABRR2*, which encode the ρ1 and ρ2 subunits of the pentameric GABA-A/GABA-C receptors. *GABRR1* and *GABRR2* lie in a head to tail orientation spanning 137 kb on chromosome 6q14-16. We genotyped 73 single nucleotide polymorphisms (SNPs), covering both genes and extending 31 kb upstream of *GABRR2* and 95 kb downstream of *GABRR1*, in a sample of 1923 European Americans from 219 multiplex alcohol-dependent families. Family-based association analyses demonstrated that SNPs in both *GABRR1* and *GABRR2* were significantly associated with alcohol dependence. Among the associated SNPs was rs282129, a coding SNP (Met430Thr) in *GABRR2*. Secondary analysis using a median split for age of onset suggests that the association is strongest when the analysis is focused upon those with earlier onset of alcohol dependence. Haplotypes in each gene were significantly overtransmitted to family members who did not meet criteria for alcohol dependence ($P < 0.04$), and a haplotype in *GABRR2* was significantly overtransmitted to family members who met a broader definition of alcoholism ($P = 0.002$) as well as DSM-IV dependence ($P = 0.04$). © 2009 Wiley-Liss, Inc.

Key words: alcoholism; GABA-A receptor; rho subunit; genetics; single nucleotide polymorphism

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INTRODUCTION

Alcoholism is a complex genetic disorder, affected by multiple genes, environmental and social influence, and interactions between genes and environmental factors [Cloninger et al., 1981; Kendler et al., 1994; Heath et al., 1997; Bierut et al., 1999]. To identify genes contributing to the risk for alcoholism, the Collaborative Study on the Genetics of Alcoholism (COGA) has utilized both linkage and association approaches, using carefully ascertained and rigorously evaluated individuals in families with multiple alcoholics [Begleiter et al., 1995; Edenberg, 2002; Foroud et al., 2000]. With this approach, COGA has provided evidence that a number of specific genes are associated with the risk for alcoholism, including *GABRA2*, *CHRM2*, *hTAS2R16*, *GABRG3*, *OPRK1*, *PDYN*, *ADH4*, *NFKB1*, *TACR3*, *NPY2R*, and *ACN9* [Dick et al., 2004; Edenberg et al., 2004, 2006, 2008; Wang et al., 2004; Hinrichs et al., 2006; Xuei et al., 2006; Dick et al., 2008; Foroud et al., 2008; Wetherill et al., 2008].

GABA-A receptors are ligand-gated anion channels formed by pentameric complexes including $\alpha, \beta, \gamma, \delta, \varepsilon, \pi$, and ρ subunits, and mediate fast synaptic inhibition in response to GABA [Cutting et al., 1991; Enoch, 2008]. GABA binds to an extracellular ligand-binding domain, while four transmembrane domains form the ion channel for chloride transportation into cells. The effects of drugs and alcohol on GABA neurotransmission have been extensively studied, as has their role in alcohol dependence [Barnard et al., 1998; Koob, 2006; Lobo and Harris, 2008; Vengeliene et al., 2008]. The GABA-ρ1 receptor subunit was originally cloned based upon homology to the GABA-A receptor subunits, and when expressed encodes a GABA-responsive chloride channel that binds mucimol [Cutting et al., 1991]. The closely related ρ2 subunit was cloned by homology to ρ1 [Cutting et al., 1992]. Sequence similarity makes them part of the GABA-A family of receptors, and they form ligand-gated chloride channels, but insensitivity of the homopentamers to baclofen and bicuculline as well as benzodiazepines lead them to also be classified as GABA-C receptors [Shimada et al., 1992; Kusama et al., 1993; Bormann, 2000; Zhang et al., 2001]. GABA-C receptors have a higher sensitivity to GABA and do not desensitize [Enz, 2001]. In the superior colliculus they appear to be involved in long-term potentiation [Enz, 2001; Schmidt et al., 2001].

Several GABA-A receptor genes are associated with alcoholism. The association of *GABRA2* with alcoholism [Edenberg et al., 2004] has been replicated in case–control studies of Germans [Fehr et al., 2006; Soyka et al., 2008], Russians [Lappalainen et al., 2005], European-Americans [Covault et al., 2004], Plain Indians [Enoch et al., 2006], and a general Australian population [Lind et al., 2008]. The association was with single nucleotide polymorphisms (SNPs) in a region of linkage disequilibrium (LD) extending from intron 3 past the 3′ end of the *GABRA2* gene [Covault et al., 2004; Edenberg et al., 2004] and may extend to the 5′ end of the adjacent *GABRG1* gene, which is in LD with SNPs in *GABRA2* [Covault et al., 2008]. In the COGA sample, the association with *GABRA2* was greatest among those alcohol-dependent individuals with comorbid dependence on illicit drugs [Agrawal et al., 2006]; this subgroup is characterized by greater severity of alcohol problems [Dick et al., 2007]. SNPs in *GABRG3* on chromosome 15 [Dick et al., 2004] and *GABRA1* on chromosome 5 [Dick et al., 2006] have also been associated with alcoholism. The evidence for associations of several GABA receptors with alcoholism, along with the known effects of alcohol on GABA neurotransmission, raises the question of whether variations in additional GABA-A receptors, including ρ receptors, might also affect risk for alcoholism.

There is evidence for linkage of the power in the gamma band (29–45 Hz) of the resting electroencephalograph (EEG) over the frontal and central scalp with markers on chromosome 6q14-16 [Xuei et al., 2008]. *GABRR1* and *GABRR2*, encoding the GABA receptor subunits ρ1 and ρ2, lie together in a 137 kb region that falls within this linkage peak. Although the ρ1 and ρ2 subunits were originally found in the bipolar neurons in the retina [Cutting et al., 1991; Polenzani et al., 1991], they are widely expressed in the brain (including cortex, thalamus, pituitary gland, cerebellum, and hippocampus) and in the spinal cord [Cutting et al., 1991; Zheng et al., 2003; Milligan et al., 2004; Lopez-Chavez et al., 2005; Alakuijala et al., 2005a,b; Harvey et al., 2006]. Homomeric ρ1 receptors are inhibited by ethanol at a low concentration (400 nM) of GABA [Mihic and Harris, 1996]. Two amino acids in the GABA-A receptor transmembrane domains 2 (Ser270) and 3 (Ala291) are critical for allosteric modulation of the receptors by alcohols and volatile anesthetics [Mihic et al., 1997]. Heteromeric complexes of ρ and α1 subunits were found in mouse cerebellar Purkinje cells, where ρ subunits contribute to functional ionotropic receptors mediating a component of phasic inhibitory GABAergic transmission [Harvey et al., 2006].

Therefore, we examined whether *GABRR1* and *GABRR2* might be associated with alcoholism. We genotyped 73 SNPs, covering *GABRR1*, *GABRR2*, and their flanking regions, in a sample of European Americans from families in which at least three first-degree relatives were alcohol dependent. We report here evidence that SNPs in both *GABRR1* and *GABRR2* were significantly associated with alcohol dependence.

MATERIALS AND METHODS

Subjects

Subjects were collected at six centers in the United States: Indiana University, State University of New York Health Science Center, University of Connecticut, University of Iowa, University of California/San Diego, and Washington University, St. Louis. The Institutional Review Boards of all participating institutions approved the study. Probands were identified through alcohol treatment programs. After providing informed consent, probands and their relatives were administered a validated poly-diagnostic instrument, the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) interview [Bucholz et al., 1994; Hesselbrock et al., 1999]. For ascertainment, alcoholism was defined as meeting criteria for both DSM-IIIR alcohol dependence [American Psychiatric Association, 1987] and Feighner definite alcoholism [Feighner et al., 1972] (this combination is called COGA criteria). Details of the ascertainment and assessment have previously been published [Begleiter et al., 1995; Reich et al., 1998; Foroud et al., 2000] and are available in detail at zork.wustl.edu/niaaa/coga_instruments/resources.html. Families in which at least three first-degree relatives

were alcohol dependent participated in the genetic part of the COGA study, as described in more detail by Foroud et al. [2000]. A sample of 1923 European American individuals from 219 alcoholic families was used in this study.

SNP Genotyping

Because HapMap data indicated that flanking genes were in LD with the *GABRR1–GABRR2* gene cluster, we extended the genotyping into the flanking regions. Seventy-three SNPs in a 262 kb region, including *GABRR1* and *GABRR2*, *UBE2J1* (ubiquitin-conjugating enzyme E2, J1) upstream of *GABRR2*, and *PM20D2* (peptidase M20 domain containing 2), *SRrp35* (serine–arginine repressor protein), and *PNRC1* (proline-rich nuclear receptor coactivator 1) downstream of *GABRR1* (Fig. 1), were selected from public databases (dbSNP and HapMap). Preference was given to SNPs having minor allele frequencies greater than 5%. Four synonymous SNPs in the coding regions of *GABRR1* and *GABRR2* (rs34218666, rs35608866, rs34617047, and rs35301635) were genotyped despite their reported low minor allele frequencies; they proved monomorphic in our sample and are not included in the tables or analyses. SNP positions were obtained from NCBI reference human genome, build 36.3.

Most assays were designed for the Sequenom MassArray system (Sequenom, San Diego, CA) using MassArray Assay Design Software. The assays were done using the hME or iPLEX assay format (Sequenom); in both cases, alleles were discriminated by mass spectrometry. Assays were tested on two groups of 40 unrelated individuals from the Coriell European-American and African-American samples. SNPs that were not in Hardy–Weinberg equilibrium in both populations were not genotyped in the sample. Some SNPs were genotyped using the Illumina technology on a BeadLab station with GoldenGate chemistry, as described previously [Dick et al., 2008].

All SNPs were tested for Mendelian inheritance using the program PEDCHECK [O'Connell and Weeks, 1998]. Marker allele frequencies and heterozygosities were computed using the program USERM13 [Boehnke, 1991]. Markers were tested for Hardy–Weinberg equilibrium in independent individuals from the study sample using Haploview [Barrett et al., 2005] and were omitted from analysis if they deviated significantly ($P < 0.001$) from Hardy–Weinberg equilibrium.

Statistical Analyses

LD among genotyped SNPs was evaluated using HAPLOVIEW [Barrett et al., 2005]. Coverage of the gene was additionally examined using Tagger [de Bakker et al., 2005] to determine the correlation between genotyped SNPs and all known SNPs in the HapMap I dataset.

Family-based association analyses were performed using the Pedigree Disequilibrium Test (PDT) [Martin et al., 2001] as implemented in the program UNPHASED (version 2.404) [Dudbridge, 2003]. The PDT uses data from all available trios in a family, as well as discordant sibships. The PDT_{sum} statistic, which gives greater weight to families with a larger number of informative trios and discordant sibships, was utilized as the primary statistic. Haplotypes from phase-certain individuals were used to further explore significant associations across the *GABRR1–GABRR2* gene

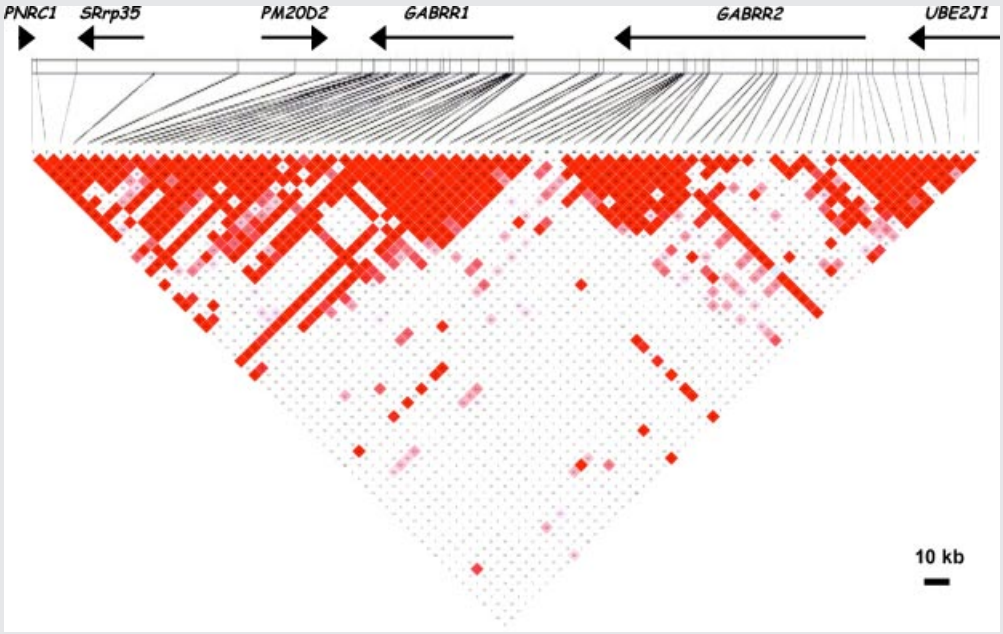

FIG. 1. Locations of genes, SNPs, and linkage disequilibrium (D′) among the SNPs. Sixty nine SNPs (MAF > 0.05) are included in the plot of D′. The location of each gene is represented proportionally across the top of the figure, with the direction of transcription represented by an arrow. The size of the gene is indicated at lower right side.

TABLE I. Phenotypic Distribution of 1,923 Genotyped Individuals

Phenotypes	# Affected (%)	# Unaffected (%)	Unknown[a] (%)
DSM-IV	718 (37.3%)	1025 (53.3%)	180 (9.4%)
COGA	862 (44.8%)	881 (45.8%)	180 (9.4%)
Early[b]	399 (20.7%)	1025 (53.3%)	499 (26.0%)

[a]There are 123 individuals without a completed SSAGA diagnostic interview, and 57 individuals recoded as unknown due to contradicting alcohol dependence classification.
[b]Subjects coded as early met the DSM-IV criteria for alcohol dependence at age 21 or younger.

cluster. Association analysis using the haplotypes was performed using UNPHASED and the PDT_{sum} statistic.

For our primary phenotype, we employed the DSM-IV criteria for alcohol dependence [American Psychiatric Association, 1994], which could be derived from the SSAGA data; we used the broader COGA definition as a secondary phenotype. The vast majority of individuals who met DSM-IV criteria also met COGA criteria; 35 individuals who were positive for DSM-IV but negative for COGA criteria and 22 individuals who were positive for COGA criteria but negative for DSM-IV were coded as unknown and omitted from analysis (Table I).

We performed secondary analyses exploring the hypothesis that genetic effects are stronger in those alcohol-dependent individuals with an earlier age of onset. Association analysis was performed defining as affected only those individuals meeting DSM-IV criteria for alcohol dependence by age 21 (n = 399; Table I). As in the above analyses of alcohol dependence, 1,025 family members were classified as unaffected. Others, including individuals meeting DSM-IV alcohol dependence criteria at age 22 or older, were considered unknown.

RESULTS

GABRR1 and *GABRR2* are in a head-to-tail orientation spanning 137 kb on chromosome 6q (Fig. 1). Based on the LD pattern, we analyzed 69 SNPs in the region extending 31 kb upstream of *GABRR2* to the adjacent gene *UBE2J1*, and 95 kb downstream of *GABRR1*; this 263 kb region included three additional genes, *PM20D2*, *SRrp35*, and *PNRC1* (Fig. 1). The mean minor allele frequency (MAF) was 0.29, with a standard deviation ±0.11 and a range from 0.06 to 0.50 (Table II). The LD pattern in our sample is similar to that in the HapMap CEU (CEPH European) database.

TABLE II. Association Analyses of *GABRR1* and *GABRR2* and Their Flanking Regions

Gene	SNP number	SNP_ID	Chromosome position[a]	SNP location[b]	MAF[c]	Minor nucleotide[d]	DSM-IV[e]	Early onset[e]	COGA[e]
PNRC1	1	rs1130809	89,850,613	Exon 2, Thr321	0.18	C	0.70	0.18[f]	0.77
	2	rs11961455	89,852,007	Downstream	0.42	C	0.66	0.23[f]	0.39
SRrp35	3	rs6938490	89,862,949	3′ UTR	0.29	A	0.90	0.47	0.33
	4	rs423516	89,884,774	Promoter	0.29	T	0.57	0.15	0.72
PM20D2	5	rs4707518	89,907,725	Upstream	0.29	C	0.69	0.22	0.98
	6	rs1929635	89,923,709	Intron 4	0.28	G	0.49	0.13	0.73
GABRR1	7	rs1331100	89,935,180	Downstream	0.29	G	0.72	0.18	0.81
	8	rs416115	89,941,956	Downstream	0.30	C	0.17	**0.014**[f]	0.08[f]
	9	rs3734201	89,945,162	3′ UTR	0.42	C	0.58	**0.04**	0.55
	10	rs1796743	89,945,463	Exon 10, Ala389	0.35	T	0.40	0.12[f]	0.27
	11	rs407221	89,947,094	Intron 8	0.27	C	0.21	0.23	0.37
	12	rs368873	89,949,881	Intron 7	0.29	G	0.43	**0.03**[f]	0.14[f]
	13	rs407206	89,951,687	Intron 7	0.32	A	0.13	0.12	0.15
	14	rs423463	89,955,427	Intron 6	0.19	A	0.68	0.41[f]	0.97
	15	rs453503	89,957,318	Intron 5	0.19	T	0.64	0.38[f]	0.66
	16	rs452667	89,960,039	Intron 5	0.19	T	0.62	1.00	0.45
	17	rs439912	89,963,734	Intron 5	0.45	C	0.13	0.56	0.32
	18	rs422751	89,964,592	Exon 5, Asp140	0.16	G	0.97	0.86	0.8
	19	rs11967322	89,966,483	Intron 3	0.43	T	0.19	0.45	0.58
	20	rs7741132	89,969,614	Intron 2	0.33	A	0.14	0.06	0.13
	21	rs7758893	89,973,360	Intron 1	0.19	C	0.79	0.51[f]	0.76
	22	rs881293	89,974,522	Intron 1	0.32	C	0.63	0.29[f]	0.71
	23	rs1029057	89,979,273	Intron 1	0.28	T	1.00	0.87	0.44
	24	rs6902106	89,982,459	Intron 1	0.28	C	0.34	0.14[f]	0.09[f]
	25	rs17504587	89,982,921	Intron 1	0.19	T	**0.04**	**0.03**	**0.01**
	26	rs1186902	89,983,681	Exon 1, Arg21His	0.28	C	0.81	0.97	0.36
	27	rs12200969	89,983,685	Exon 1, Val20Met	0.34	C	0.20	0.09[f]	0.11[f]
	28	rs1186903	89,984,290	Promoter	0.35	A	0.11[f]	**0.048**[f]	0.10[f]

[*Continued*]

TABLE II. (*Continued*)

Gene	SNP number	SNP_ID	Chromosome position[a]	SNP location[b]	MAF[c]	Minor nucleotide[d]	DSM-IV[e]	Early onset[e]	COGA[e]
	29	rs914479	89,986,927	Upstream	0.35	C	0.08[f]	**0.028**[f]	**0.04**[f]
	30	rs914478	89,987,638	Upstream	0.34	C	0.12[f]	0.06[f]	0.07[f]
	31	rs9342188	90,002,245	Intergenic	0.09	C	0.83	0.27[f]	0.59
	32	rs1321355	90,007,632	Intergenic	0.27	G	0.10[f]	0.10[f]	0.08[f]
	33	rs282135	90,008,949	Intergenic	0.28	G	0.74	0.93	0.24
	34	rs6914006	90,014,480	Intergenic	0.44	A	0.46	0.47	0.73
GABRR2	35	rs3734198	90,021,011	Downstream	0.43	G	0.62	0.84	0.92
	36	rs282129	90,024,217	Exon 9, Met430Thr	0.28	A	**0.03**[f]	0.08[f]	**0.02**[f]
	37	rs13211104	90,027,125	Intron 8	0.30	A	**0.03**[f]	0.07[f]	**0.03**[f]
	38	rs9451191	90,029,831	Intron 8	0.26	A	**0.021**	**0.002**	0.17
	39	rs723041	90,030,782	Intron 8	0.13	T	0.79	0.86	0.83
	40	rs9294426	90,031,297	Intron 7	0.29	A	0.55	0.38	1.00
	41	rs12206367	90,031,345	Intron 7	0.12	A	0.43	0.38	0.84
	42	rs2273507	90,031,964	Intron 7	0.20	G	0.70	0.55	0.52
	43	rs2273508	90,034,567	Intron 4	0.11	T	0.97	0.63	0.46
	44	rs282121	90,036,802	Intron 3	0.41	T	**0.025**	**0.02**	**0.05**
	45	rs282117	90,038,132	Exon 3, Val83	0.42	T	0.07	**0.017**	0.12
	46	rs282115	90,038,431	Intron 2	0.07	A	0.78	0.20[f]	0.96
	47	rs9451194	90,042,098	Intron 2	0.45	A	0.91	0.53	0.65
	48	rs9362632	90,051,417	Intron 2	0.29	G	0.87	0.88	0.36
	49	rs3798256	90,053,124	Intron 2	0.45	G	0.22	0.06	0.36
	50	rs964626	90,056,015	Intron 2	0.21	C	0.13[f]	0.16[f]	0.13[f]
	51	rs9451196	90,057,273	Intron 2	0.12	T	0.07[f]	0.21[f]	0.30[f]
	52	rs6454750	90,057,490	Intron 2	0.45	T	1.00	0.65	0.80
	53	rs2148174	90,062,644	Intron 2	0.33	T	0.41	0.78	0.85
	54	rs7742664	90,065,463	Intron 2	0.22	T	0.20[f]	**0.03**[f]	0.25[f]
	55	rs2325202	90,068,229	Intron 1	0.36	G	0.22	**0.04**[f]	0.16
	56	rs1570028	90,069,154	Intron 1	0.49	A	0.34	0.07[f]	0.08
	57	rs6942204	90,072,687	Intron 1	0.27	C	**0.04**	**0.005**	**0.016**
	58	rs6454752	90,075,080	Intron 1	0.23	T	0.43	0.21[f]	0.07[f]
	59	rs9294432	90,076,686	Intron 1	0.47	T	0.51	0.14[f]	**0.04**[f]
	60	rs10944441	90,078,260	Intron 1	0.18	A	0.08[f]	**0.012**[f]	**0.005**[f]
	61	rs7764923	90,079,640	Intron 1	0.24	T	0.08[f]	**0.02**[f]	**0.007**[f]
	62	rs2236204	90,081,831	Promoter	0.19	T	0.09[f]	**0.015**[f]	**0.006**[f]
	63	rs9362636	90,083,516	Promoter	0.06	G	0.86	0.88	0.91
	64	rs2064831	90,089,661	Upstream	0.18	C	0.09[f]	**0.01**[f]	**0.009**[f]
UBE2J1	65	rs13215418	90,093,230	Downstream	0.17	T	0.56	0.93	0.73
	66	rs10502	90,096,389	Exon 8, Val229Leu	0.40	C	0.34	0.11[f]	0.07[f]
	67	rs12189673	90,103,123	Intron 5	0.30	C	0.62	0.25	**0.05**
	68	rs7760851	90,109,323	Intron 2	0.50	G	0.40	0.48	**0.009**
	69	rs7743444	90,112,800	Intron 1	0.24	T	0.88	0.97	0.52

[a]Chromosome positions are based on NCBI Human Genome Assembly v36.3.
[b]*PNRC1* and *PM20D2* are transcribed in forward direction; *SRrp35*, *GABRR1*, *GABRR2*, and *UBE2J1* are transcribed in the opposite direction.
[c]Minor allele frequency in this sample of European Americans.
[d]Nucleotides are shown on the human genome strand.
[e]*P*-value of UNPHASED PDT_{sum} statistic for association between the SNP and alcohol dependence. $P \leq 0.05$ is in bold.
[f]Minor allele is preferentially transmitted to affected individuals.

SNP coverage was further assessed using Tagger [de Bakker et al., 2005]; 61 of the SNPs were included in the HapMap database and could, therefore, be analyzed. In the CEU population, these 61 SNPs gave a mean r^2 of 0.79 with a total of 250 HapMap SNPs in the region; 65% of the SNPs in the region were captured at $r^2 \geq 0.8$ and 84% at $r^2 \geq 0.5$. The eight additional SNPs we genotyped that were not included in the HapMap database would further increase the coverage of this region. Thus, the SNPs we genotyped provide very good coverage of the variation in the *GABRR1–GABRR2* gene cluster and adjacent genes.

Six SNPs were significantly associated with alcohol dependence defined by DSM-IV criteria ($P < 0.05$) (Table II, Fig. 2); five of these were located in *GABRR2* from intron 1 to exon 9 and 1 was in intron 1 of *GABRR1*. Interestingly, rs282129 ($P = 0.03$) is a nonsynonymous coding SNP that leads to either a methionine or a threonine at amino acid 430 of *GABRR2*; the allele overtransmitted to the

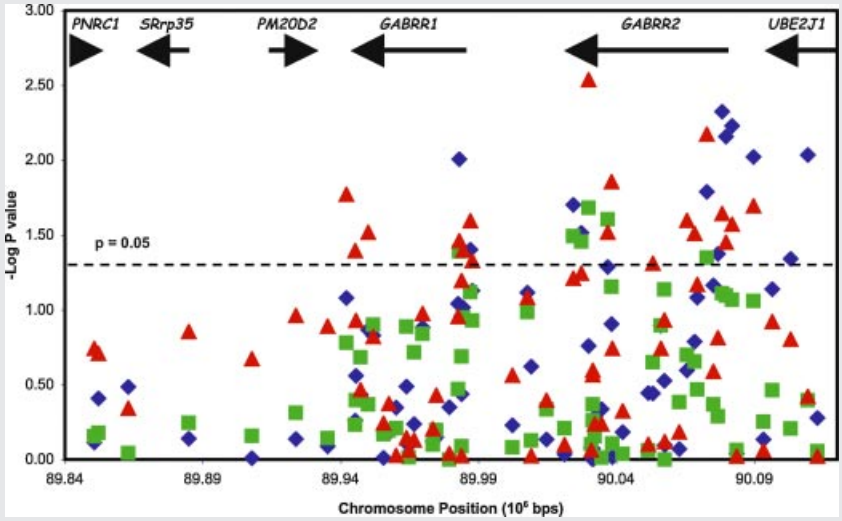

FIG. 2. Association of *GABRR1* and *GABRR2* with alcohol dependence. The −log (*P*-value) of the PDT_{sum} statistic for each SNP is plotted as a function of chromosomal position (10^6 bp). The solid squares represent alcohol dependence using DSM-IV criteria; the solid triangles represent early onset alcohol dependence; the solid diamonds represent alcohol dependence using COGA criteria. Dashed line indicates *P*-value = 0.05. The gene locations are shown across the top.

affected individuals encodes methionine. Eight additional SNPs (including four in the promoter to intron 1 region of *GABRR2* and 1 upstream of *GABRR1*) demonstrated suggestive association ($0.05 < P \leq 0.10$).

We have previously found that the associations of *GABRA2*, *CHRM2*, and *NFKB1* with alcoholism were strongest in the half of the sample that included the most severely affected individuals, whose onset of alcohol dependence was at age 21 or younger [Agrawal et al., 2006; Dick et al., 2007; Edenberg et al., 2008]. Therefore, we performed additional analysis using a median split for age of onset of alcohol dependence, defining affected individuals as those who met DSM-IV criteria for alcohol dependence at age 21 or earlier (n = 399). Despite the reduction in power due to the substantially lower number of affected individuals, greater evidence for association was observed, with 16 significantly associated SNPs ($P \leq 0.05$) in the *GABRR1* and *GABRR2* gene cluster, including a synonymous SNP (Val83; rs282117) in *GABRR2*, and another 8 suggestive ($0.05 < P \leq 0.10$; Table II, Fig. 2).

Secondary analyses using the broader COGA criteria for alcohol dependence provided evidence for association; a total of 13 SNPs, including 8 concentrated in the region from intron 1 of *GABRR2* upstream to a part of *UBE2J1* that was in LD with the 5′ region of *GABRR2*, and 2 in the region from just upstream to intron 1 of *GABRR1* (Table II, Fig. 2).

The pattern of LD was examined in each of the regions having SNPs associated with at least one of the three phenotypes (DSM-IV alcohol dependence, early onset DSM-IV alcohol dependence, and COGA alcohol dependence). While there was extensive LD as defined by D′ (Fig. 1), differences in allele frequencies mean that the correlation of the SNPs (r^2) was much more restricted (Fig. 3), such that different groups of SNPs were correlated with each other but much less with other groups. Considering the LD pattern, it appears that there are at least six independent regions of association. Several groups of SNPs in LD with each other show similar patterns of association. At the 3′ end of *GABRR1*, SNPs 8, 10, and 12 are in high LD with each other (Fig. 3, inset) and two provide evidence of association with early onset, while the third, less correlated SNP, approaches significance; SNP 9, in that cluster, is also significant. At the 5′ end of *GABRR1*, three SNPs (25, 28, 29) are significantly associated with early onset (and several others are nearly significant), with some evidence for association with the COGA definition of dependence (Table II, Fig. 3). Three SNPs (36–38) at the 3′ end of *GABRR2* (which is just upstream of the 5′ end of *GABRR1*, Fig. 3) show evidence for association with all three phenotypes, particularly DSM-IV dependence. Two of these, including the cSNP at position 430, are in LD with the group of SNPs at the 5′ end of *GABRR1* (28–32; Fig. 3, inset). Thus these two groups of SNPs may represent a single finding. SNP 38 is not in LD with other SNPs, and is most significant for the early onset phenotype.

One SNP from each of these latter two groups was selected (rs914479 and rs282129; $r^2 = 0.42$) and used in haplotype analysis to test whether a high risk haplotype could be identified within the region spanned by SNPs 27–37. The global test of significance was significant for all three alcohol dependence phenotypes: DSM-IV ($P = 0.016$), COGA ($P = 0.014$), and early onset ($P = 0.030$) (Table IIIa). For all three phenotypes, a common haplotype having a frequency of 61% (T-G) was associated with the absence of alcohol dependence $P < 0.008$ for DSM-IV; $P < 0.007$ for COGA; $P = 0.019$ for early onset. The complementary haplotype C-A, consisting of the minor alleles of both SNPs, was associated with COGA alcohol dependence ($P = 0.02$) and showed a trend toward significant association with DSM-IV ($P = 0.11$); the low frequency of this haplotype gives it lower power.

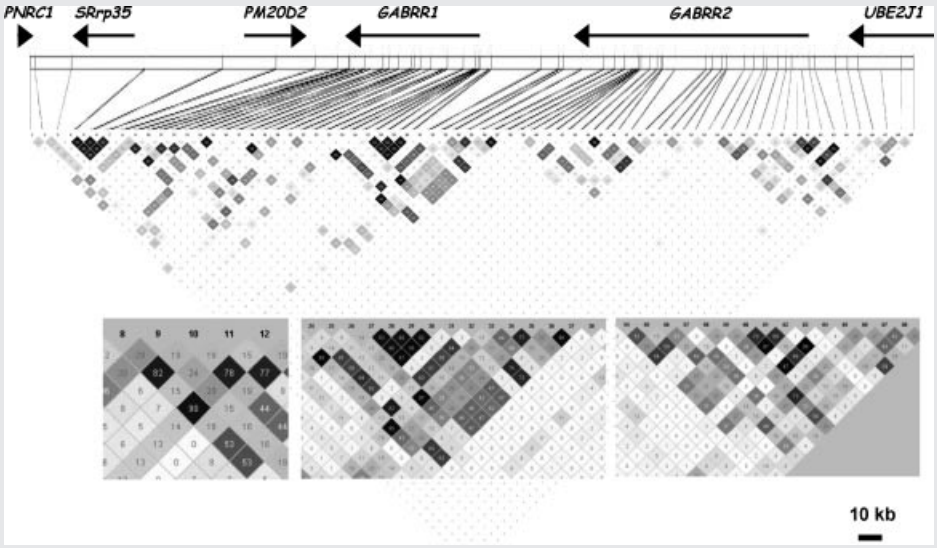

FIG. 3. Linkage disequilibrium (r^2) among the SNPs. The correlation among SNPs (r^2) across the region is shown in the large figure; insets magnify three regions discussed in the text.

There are two other clusters of SNPs in *GABRR2* (SNPs 44–49 and 54–64) with high LD within each group ($r^2 > 0.54$ and $r^2 > 0.64$ respectively) but little LD between the two groups. There is consistency in the phenotypes associated with the significant SNPs and with the pattern of LD. One SNP from each group was selected for haplotype analysis (rs282121 and rs10944441; $r^2 = 0.06$). The global test of association was significant for the broader COGA phenotype ($P = 0.005$) and for early onset ($P = 0.05$) alcohol dependence, and marginally significant for DSM -IV alcohol dependence ($P = 0.07$) (Table IIIb). Haplotype T -G (frequency 38%), consisting of the minor allele of rs282121 and the major allele of rs10944441, was overtransmitted to individuals not meeting any of the three alcohol dependence criteria and its complement, C-A (frequency 12%), was overtransmitted to the individuals meeting COGA ($P = 0.002$) and DSM-IV ($P = 0.044$) criteria for alcohol dependence, but not related to early onset.

DISCUSSION

This is the first study reporting evidence of association between *GABRR1* and *GABRR2* and alcohol dependence. Our primary analysis, using DSM-IV criteria as the phenotype, gave evidence of association primarily in *GABRR2* (five significant and six suggestive SNPs; Fig. 1, Table II). Among the associated SNPs, rs282129 is a nonsynonymous coding SNP in exon 9 of *GABRR2*; the major allele (G) encodes Thr430 in transmembrane domain 4, which forms part of the ion channel, and the minor allele (A) encodes Met430 (nucleotides are shown on the human genome strand). Met is overtransmitted to alcoholic individuals, suggesting that the protein with Met430 might differ in function in a way that could contribute to the development of alcohol dependence. However, this cSNP is in a larger region of LD extending to the upstream region of *GABRR1*, thus it could be that other regulatory variations in the region are responsible for the association.

TABLE IIIa. Haplotype Analysis of 5′ Region of *GABRR1* and 3′ Region of *GABRR2*

rs914479 nucleotide[a]	rs282129 nucleotide[a]	DSM-IV[c]	Early onset[c]	COGA[c]
T	G	**0.008**	**0.019**	**0.007**
T	A[b]	0.08	0.07	0.43
C[b]	G	0.33	0.12	0.34
C[b]	A[b]	0.11	0.35	**0.023**
Global test		**0.016**	**0.03**	**0.014**

[a]Nucleotides are shown on the human genome strand.
[b]Minor allele.
[c]*P*-value of UNPHASED PDT_{sum} statistic for association between the haplotype and alcohol dependence. $P \leq 0.05$ is in bold.

TABLE IIIb Haplotype Analysis of *GABRR2*

rs282121 nucleotide[a]	rs1094441 nucleotide[a]	DSM-IV[c]	Early onset[c]	COGA[c]
T[b]	A[b]	0.84	0.30	0.24
T[b]	G	**0.035**	**0.014**	**0.012**
C	A[b]	**0.044**	0.17	**0.002**
C	G	0.37	0.25	0.67
Global test		0.07	**0.05**	**0.005**

[a]Nucleotides are shown on the human genome strand.
[b]Minor allele.
[c]*P*-value of UNPHASED PDT_{sum} statistic for association between the haplotype and alcohol dependence. $P \leq 0.05$ is in bold.

Because early onset of alcoholism often reflects greater severity, including a higher risk for antisocial personality disorder and conduct disorder [Dick et al., 2007], and some of our earlier findings were stronger with this sub-phenotype [Agrawal et al., 2006; Dick et al., 2007; Edenberg et al., 2008], we examined whether the findings were primarily due to the cases with early onset. This was, in fact, found: 18 SNPs spread across the 2-gene cluster, including a synonymous coding SNP (rs282117; Val83) in *GABRR2*, were significantly associated with early onset alcohol dependence. The increase in evidence for association despite the reduction by nearly half in the number of individuals deemed affected in this analysis suggests that the association with alcohol dependence is particularly strong in individuals who developed alcoholism at age 21 or younger.

Secondary analysis using a broader definition of alcoholism that includes additional individuals (COGA definition; Table I) shows more SNPs significantly associated, particularly in the 5′ portion of *GABRR2*. The associated SNPs within each portion of *GABRR1* and *GABRR2* are in LD with each other (Fig. 3). Both regions of association cover the 5′ promoter to the intron 1 region of each gene, suggesting that they may affect the regulation of these genes. Several SNPs in the two associated regions (SNPs 25–27 and 36, and SNPs 60–62) are located in highly conserved regions, suggesting potential functional roles in gene expression. The two intronic SNPs in *UBE2J1* (SNPs 67 and 68) are in LD with the upstream region of *GABRR2* (Fig. 3), and may also affect expression of *GABRR2*.

The pattern of LD across these genes suggests that there are at least six different regions of association (Fig. 3), and that there may be several different susceptibility haplotypes in this region. Between the 5′ region of *GABRR1* and the 3′ end of *GABRR2*, we detected a haplotype (rs914479[T]–rs282129[G]) that appears to confer protection against all three phenotypes of alcohol dependence (Table III). This region includes the coding SNP rs282129. A haplotype within the coding and proximal 5′ portion of *GABRR2* (rs282121[T]–rs1094441[G]) also appears to confer protection from alcohol dependence (Table III). We detected strong evidence of association of the complementary haplotype rs282121[C]–rs1094441[A] to COGA criteria for alcohol dependence ($P = 0.002$), less evidence of association with the narrower DSM-IV criteria ($P = 0.044$) but no evidence of association with the early onset subset of our sample; this pattern suggests that the C-A haplotype confers risk for alcoholism but not particularly for the more severe manifestations.

We analyzed one primary phenotype, DSM-IV alcohol dependence, and have performed secondary analyses using two phenotypes that included as affected only those meeting criteria for early onset DSM-IV alcohol dependence or those meeting the broader phenotype of alcohol dependence by COGA criteria. These alcohol dependence phenotypes are largely nested; they are not independent phenotypes, and some of the same SNPs affect risk for more than one of them. There is consistency in the patterns of association among the SNPs in LD with each other.

In summary, we have found evidence for association between *GABRR1* and *GABRR2* and alcohol dependence in a family-based study, with most of the evidence coming from individuals with early onset of the disorder. This report further supports the role of GABA receptor systems in the involvement of neurotransmission in response to alcohol. It may also provide future directions for functional studies of GABA ρ receptors.

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