American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 117B:39–45 (2003)

Association of $GABA_A$ Receptors and Alcohol Dependence and the Effects of Genetic Imprinting

Jiuzhou Song,[1] Daniel L. Koller,[2] Tatiana Foroud,[2] Kristie Carr,[1] Jinghua Zhao,[1] John Rice,[3] John I. Nurnberger Jr,[2] Henri Begleiter,[4] Bernice Porjesz,[4] Tom L. Smith,[5] Marc A. Schuckit,[5] and Howard J. Edenberg[1,2]*

[1]*Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, Indiana*
[2]*Department of Medical and Molecular Genetics, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, Indiana*
[3]*Washington University, St. Louis, Missouri*
[4]*State University of New York, Health Sciences Center, Brooklyn, New York*
[5]*University of California at San Diego, San Diego, California*

GABA receptor genes have been postulated as candidates affecting the risk for alcoholism. The potential association between genes encoding five subunits of the $GABA_A$ receptors and alcoholism (alcohol dependence) was analyzed in the multiplex alcoholic pedigrees collected by the Collaborative Study on the Genetics of Alcoholism (COGA) using family-based association tests. We found consistent, although weak, linkage disequilibrium between *GABRB1* (located on chromosome 4) and alcoholism ($P < 0.03$). Genes encoding *GABRA1* and *GABRA6*, on chromosome 5, did not provide evidence for association with alcoholism. *GABRA5* and *GABRB3*, on chromosome 15, were reported to be expressed uniparentally from the paternal chromosome. Analyses of paternal transmission of alleles of *GABRA5* provided evidence for association with alcoholism, particularly in the Caucasian population and with the stricter ICD-10 definition of alcoholism ($P < 0.004$). Evidence of association was also observed during paternal transmission with *GABRB3* in the Caucasian population ($P < 0.007$). Maternal transmissions provided no evidence for association. These data are consistent with an association between the expressed alleles in the $GABA_A$-gene cluster on chromosome 15 and alcoholism that is modulated by genetic imprinting.

KEY WORDS: alcoholism; gamma-aminobutyric acid receptor; genetic imprinting; genetic association

INTRODUCTION

Alcoholism (alcohol dependence) is a clinically and genetically heterogeneous disease. Among its symptoms are 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]. Both genetic and environmental factors contribute to the risk for alcoholism. Family, adoption, and twin studies provide convergent evidence for hereditary factors in alcoholism [Goodwin, 1979; Pickens et al., 1991; Kendler et al., 1994; Heath et al., 1997]. Heritable influences account for ~40–60% of the total variance in risk [Pickens et al., 1991; Kendler et al., 1994; Heath et al., 1997].

The principal inhibitory neurotransmitter in the vertebrate brain is γ-aminobutyric acid (GABA) [Barnhard et al., 1998]. Binding of GABA to ionotropic $GABA_A$ receptors causes the opening of an integral chloride-ion channel, thus changing the membrane potential of neurons and thereby exerting a crucial role in regulating brain excitability. $GABA_A$ receptors

Grant sponsor: National Institute on Alcohol Abuse and Alcoholism (NIAAA) (NIH grant); Grant numbers: U10AA08403, AA00285.

Jiuzhou Song's present address is Department of Biochemistry and Molecular Biology, University of Calgary Faculty of Medicine, 3330 Hospital Dr. NW, Calgary, AB T2N 4N1, Canada.

*Correspondence to: Dr. Howard J. Edenberg, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202-5122.
E-mail: edenberg@iupui.edu

Received 22 February 2002; Accepted 29 August 2002
DOI 10.1002/ajmg.b.10022

are sensitive to ethanol and are believed to mediate many of its effects, including anxiolysis, sedation, motor incoordination, tolerance, and dependence [Grobin 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 due to the presence of receptors composed of different groups of subunits [Barnhard et al., 1998; Grobin et al., 1998]. A variation in the *Gabrg2* gene in mice correlates with the severity of alcohol withdrawal [Buck and Hood, 1998]. These lines of evidence suggest that variations in the GABA receptor genes contribute to differences in risk for alcoholism.

$GABA_A$ receptors are pentameric assemblies of subunits; 19 mammalian subunits are known, which are classified into α, β, γ, δ, ε, π, and ρ types [Barnhard et al., 1998]. Most GABA receptors contain α, β, and γ subunits [Barnhard et al., 1998; Sieghart et al., 1999]. Most of the genes encoding human $GABA_A$ receptor subunits are organized in clusters. *GABRA2*, *GABRA4*, *GABRB1*, and *GABRG1*, encoding the α2, α4, β1, and γ1 subunits, are on chromosome 4p12 [Wilcox et al., 1992; McLean et al., 1995; Bailey et al., 1999; Russek, 1999] (NCBI LocusLink). *GABRA1*, *GABRA6*, *GABRB2*, and *GABRG2*, encoding the α1, α6, β2, and γ2 subunits, respectively, are localized to chromosome 5q34 [Wilcox et al., 1992; Bailey et al., 1999; Russek, 1999] (NCBI LocusLink). *GABRA5*, *GABRB3*, and *GABRG3*, encoding the α5, β3, and γ3 subunits, are on chromosome 15q11.2-q12 [Sinnett et al., 1993; Greger et al., 1995; Glatt et al., 1997; Christian et al., 1998; Russek, 1999] (NCBI LocusLink). The clustering of GABA-receptor subunit genes might have functional significance. Uusi-Oukari et al. [2000] reported that altering the structure of the mouse *Gabra6* affects expression of the other genes in the cluster, *Gabra1* and *Gabrb2*.

There have been several studies of the potential association of genes encoding $GABA_A$ receptor subunits with alcoholism. Parsian and Cloninger [1997] examined microsatellite polymorphisms in *GABRA1* and *GABRA3* in a sample of alcoholics and controls of Western European descent, and found no significant association with alcoholism or with type I and type II subsets of alcoholics. Parsian and Zhang [1999] found association between a microsatellite polymorphism in the *GABRB1* gene and alcoholism in the same population. There have been several papers examining the gene cluster on chromosome 5. Sander et al. [1999] examined single nucleotide polymorphisms (SNPs) in *GABRA6*, *GABRB2*, and *GABRG2* in 349 German alcoholics and 182 ethnically matched controls, and found no significant association with alcohol dependence or withdrawal or familial alcoholism. Loh et al. [2000] carried out association studies of five polymorphisms in GABA subunit genes on chromosome 5 in Japanese, and found no association of any with alcoholism, or alcoholism without concurrent antisocial personality disorder, but a marginal association of one polymorphism in *GABRG2* for alcoholism with antisocial personality disorder ($P = 0.021$). In a Scottish population, Loh et al. [1999] reported associations between alcoholism and polymorphisms in *GABRA6* and *GABRB2*. Loh and Ball [2000] reviewed studies on the chromosome 5 cluster and argued for its relevance to alcohol dependence, but there are more negative or equivocal results than positive. Iwata et al. [2000] found a Pro385Ser variant at low frequency in *GABRA6*; there was no significant association of this variant with alcoholism in a Finnish population. A preliminary report by Schuckit et al. [1999] suggested that there was an association between this Pro385Ser polymorphism and alcoholism. These mixed results could be due to the small sample sizes and differences among populations.

Meguro et al. [1997] examined the expression of the GABA-receptor genes clustered on chromosome 15. They constructed hybrid cells in which a single human chromosome 15 was contained in mouse A9 cells. This system maintained the known paternal-specific expression of the imprinted genes *SNRPN* and *IPW*. In this system, they demonstrated that *GABRB3*, *GABRA5*, and *GABRG3* were expressed exclusively from the paternal allele [Meguro et al., 1997]. They also showed hypermethylation of the paternal allele of *GABRB3*. LaSalle and Lalande [1995] demonstrated allele-specific replication timing in the region containing the *GABRB3* and *GABRA5* genes.

Here we report family-based analyses of the potential association of alcoholism with $GABA_A$ receptor genes located on three chromosomes. Family based association studies avoid the problems of false positive results due to population stratification, which can occur in population based association approaches. The sample used in the study, collected by the COGA, consists of systematically ascertained families with multiple alcoholic members. Families with multiple affected individuals are more likely to have an increased number of genetic risk factors for alcoholism. Because Meguro et al. [1997] showed imprinting (uniparental expression, from the paternal chromosome) of the $GABA_A$ receptor genes on chromosome 15, we examined paternal transmission of alleles of *GABRB3* and *GABRA5*.

METHODS

Subjects

Probands from alcohol treatment facilities who met both a lifetime diagnosis of alcohol dependence by DSM-III-R criteria [American Psychiatric Association, 1987] and definite alcoholism by the Feighner criteria [Feighner et al., 1972] were recruited to participate in the COGA. The combination of DSM-III-R and Feighner criteria (called COGA criteria) identifies individuals who are clearly alcohol dependent, and allows comparability with earlier studies. Details of the ascertainment and characterization have been reported [Bucholz et al., 1995; Foroud et al., 1998]. Probands and all available family members were extensively interviewed (after informed consent was obtained) using the semi-structured assessment for the genetics of alcoholism (SSAGA) [Bucholz et al., 1994, 1995]. The SSAGA provides data from which diagnoses by criteria from other systems, including DSM-III-R [American Psychiatric Association, 1987], Feighner [Feighner et al., 1972], and

ICD-10 [World Health Organization, 1993], can be derived. Families with at least three first-degree relatives meeting the COGA criteria for alcoholism were entered into the genetic part of the study; use of other drugs was not grounds for exclusion, except probands were excluded for intravenous drug use [Reich et al., 1998]. Extended families were rigorously evaluated to identify alcoholic individuals. As a result, pedigrees often included some interviewed branches that did not include alcoholic individuals. Thus, to reduce the extent of genotyping only the genetically most informative families were included in the genotyping portion of the study. In addition, only individuals from that portion of the pedigree that included alcoholic members were genotyped. The study was approved by the appropriate institutional review boards of participating institutions.

Genotyping and Data Cleaning

Microsatellite (simple sequence repeat) polymorphisms were determined as previously described [Reich et al., 1998; Foroud et al., 2000]. Primer sequences used for the dinucleotide repeat of *GABRB1* (CGTAAGCGTGCACTATACCCT and GCTGAGGATTCATCCACCTG; GABRB1-1 and GABRB1-2. All primers are listed 5′ to 3′) amplify 85–103 bp DNA fragments (The Genome Database, http://www.gdb.org/). *GABRA1* fragments from 180 to 208 bp were amplified with primers GCCAACATCTGATGTTCAC and GGAAATGAAGATTAACCAGC [Johnson et al., 1992]. The *GABRB3* dinucleotide repeat produced fragments from 181 to 201 bp, using CTCTTGTTCCTGTTGCTTTCAATACAC and GCACTGTGCTAGTAGATTCAGCTC primers [Mutirangura et al., 1992]. The *GABRA5* dinucleotide repeat used primers GATGACTTACCCACCTTTATTC and GTAGAATTTCCCTGTAAAGGCAC to produce fragments of 278–290 bp [Glatt et al., 1992]. Data were independently checked and entered by two individuals. The results were compared and any discrepancies were reexamined. Genotypes without remaining discrepancies were forwarded to the database.

Genotyping of the *GABRA6* Pro386Ser polymorphism was performed using allele-specific oligonucleotides after PCR amplification [Schuckit et al., 1999]. Briefly, a 365 bp DNA fragment was amplified using CTGACTCCAAATATCATATG and GAGAAGCATCTACACAAGTC as primers. The reaction mixture contained 150 ng of genomic DNA, 20 pmol of each primer, 2.5 mM each dNTP, Taq polymerase, and buffer (Perkin Elmer/Cetus, Norwalk, CT). Cycling was for 5 min at 93°C, followed by 35 cycles of 94°C (20 s), 56°C (10 s), and 72°C (25 s), and then 5 min at 72°C. PCR products were denatured and transferred to 0.45 μm Zeta-Probe nylon membranes (Bio-Rad, Hercules, CA) using a dot blot apparatus. Allele-specific oligonucleotide probes CCTGTCACA***C***CCCCACCAC and CCTGTCACA***T***CCCCACCAC were labeled with ^{32}P using T4 polynucleotide kinase and separately hybridized overnight. Membranes were washed successively at 58, 60, and 62°C. Bound radioactivity was determined using a BioRad phosphorimager.

A specially constructed database, GeneMaster (J. Rice), was used to check for alleles in an individual not present in his/her parents; discrepancies were re-examined. If data remained incompatible after review, the genotypic data from one or more individuals incompatible with the rest of the family were removed. Data cleaning was done blind to affected status. Additional data cleaning and allele frequency estimation were performed using USERM13 [Boehnke, 1991].

Statistical Methods

The data from these candidate genes were analyzed using the transmission disequilibrium test (TDT) [Spielman et al., 1993; Spielman and Ewens, 1996], which tests for unequal transmission of alleles from heterozygous parents to their affected offspring. Calculations were performed using the TDTEX component of S.A.G.E. Two hierarchical definitions of alcoholism were utilized: 1) the COGA criteria (both DSM-III-R alcohol dependence and Feighner definite alcoholism [Feighner et al., 1972; American Psychiatric Association, 1987]), and 2) the narrower ICD-10 criteria for alcohol dependence [World Health Organization, 1993]. In the COGA sample, individuals who meet the ICD-10 criteria are a subset of those who meet the COGA criteria [Foroud et al., 1998]. Analysis was performed in the total sample and then separately in the non-Hispanic Caucasian subset (~80% of the total). Other ethnic groups were represented in numbers too low to allow separate analysis.

The selection of the single affected offspring in each pedigree was based on heterozygosity of the parental genotypes for each gene examined, as previously described [Edenberg et al., 1998]. The marginal test evaluates the evidence of disequilibrium by comparing the marginal totals for each allele (transmitted and non-transmitted) in the transmission table. In this case, if there is increased or decreased transmission of a particular allele, it is considered evidence in favor of disequilibrium. We focused on the permutation-based *P*-value from each test, rather than the asymptotic *P*-values, because of the sparseness of some cells due to rare alleles.

Because a previous study [Meguro et al., 1997] suggested that only the paternal alleles of the $GABA_A$ receptor genes on chromosome 15 are expressed, we examined transmission of paternal alleles of *GABRB3* and *GABRA5*.

RESULTS

TDT analyses of five genes encoding $GABA_A$ receptor subunits were conducted to explore whether there was evidence for linkage disequilibrium between these genes and alcoholism. The most consistent finding was modest linkage disequilibrium between alleles at the *GABRB1* locus on chromosome 4 and alcohol dependence as defined either by COGA ($P < 0.03$) or ICD-10 criteria ($P < 0.03$) (Table I). This was also true when the sample was limited to the non-Hispanic Caucasian subset (COGA: $P < 0.02$).

GABRA1 and *GABRA6*, clustered on chromosome 5, provided no evidence of linkage disequilibrium with

TABLE I. GABRB1 Association With COGA and ICD-10 Diagnoses

	COGA				ICD-10			
	Combined		Caucasian		Combined		Caucasian	
Allele	Trans[a]	Not[a]	Trans	Not	Trans	Not	Trans	Not
93	11	28	10	24	9	25	8	21
95	54	66	51	64	44	54	42	52
97	86	57	82	51	73	47	68	42
99	35	37	30	35	30	33	25	30
101	4	2	3	2	4	1	3	1
Total	190	190	176	176	160	160	146	146
P value	0.03		0.02		0.03		0.02	

[a]TRANS, transmitted to offspring; NOT, not transmitted, numbers of alleles either transmitted or not transmitted from heterozygous parents.

either definition of alcoholism. This lack of evidence held for either the entire sample or the Caucasian subset, and for both definitions of alcohol dependence ($P \leq 0.20$; data not shown).

The cluster of $GABA_A$ receptor genes on chromosome 15 provided interesting results. There was modest evidence for linkage disequilibrium between the *GABRA5* and alcoholism as defined by ICD-10 criteria ($P < 0.03$, Table II) in the Caucasian subset, but only a trend in the overall data ($P < 0.09$) (Table II). *GABRB3* showed no evidence of association when analyzed in this way ($P > 0.12$). There was a previous report of parental imprinting of the $GABA_A$ receptor genes in this region of chromosome 15, with only the paternal allele expressed [Meguro et al., 1997]. This imprinting would make the paternal gene the only one that could influence the phenotype; the silent maternal gene should not have any effect. Therefore, we separately analyzed transmission of the paternal (active) alleles (Tables II, III). *GABRB3* showed significant association with alcoholism as defined by both criteria, COGA and ICD-10, in the overall dataset, ($P = 0.04$ for ICD-10; $P = 0.03$ for COGA, Table III), and stronger association when the Caucasian only subset was analyzed ($P = 0.007$ for ICD10, $P = 0.006$ for COGA). *GABRA5* showed striking association with the narrower ICD-10 criteria for the full and Caucasian-only datasets ($P = 0.008$, $P = 0.004$, respectively). There was no significant association with maternal transmissions, which were analyzed as a control ($P > 0.37$ for all comparisons).

TABLE II. GABRB3 and GABRA5 Association With ICD-10 Diagnosis

	Total sample						Caucasians only					
	All		Paternal		Maternal		All		Paternal		Maternal	
Allele	Trans	Not	Trans	Not	Trans	Not	Trans	Not	Trans	Not	Trans	Not
GABRB3												
182	43	62	19	32	19	23	41	59	19	30	17	22
184	15	15	10	8	4	5	13	13	8	6	4	5
186	22	19	15	8	5	9	20	17	14	8	4	7
188	19	11	6	6	12	5	13	10	4	5	8	5
190	12	7	4	2	7	4	10	6	4	1	5	4
192	7	7	5	3	1	4	5	1	4	1	0	0
194	41	39	16	18	19	16	36	37	12	18	18	14
196	10	4	6	3	4	1	10	3	6	2	4	1
198	18	15	12	5	5	8	18	13	12	4	5	7
200	3	11	0	8	2	3	3	10	0	8	2	2
Total	190	190	93	93	78	78	169	169	83	83	67	67
P value	0.20		0.04		0.42		0.12		0.007		0.61	
GABRA5												
278	6	5	1	1	3	4	5	3	1	1	3	2
280	7	5	4	3	2	2	7	5	4	2	2	2
282	15	25	5	13	6	8	15	25	5	13	6	8
284	19	22	7	10	9	6	16	20	6	9	7	6
286	10	14	3	7	4	4	8	14	2	7	3	4
288	21	8	14	1	5	5	21	7	14	1	5	4
290	2	1	1	0	1	1	2	1	1	0	1	1
Total	80	80	35	35	30	30	74	74	33	33	27	27
P value	0.09		0.008		0.98		0.03		0.004		0.99	

TABLE III. GABRB3 and GABRA5 Association With COGA Diagnosis

	Total sample						Caucasians only					
	All		Paternal		Maternal		All		Paternal		Maternal	
Allele	Trans	Not	Trans	Not	Trans	Not	Trans	Not	Trans	Not	Trans	Not
GABRB3												
182	48	74	20	38	23	27	47	70	20	36	22	25
184	17	21	11	9	4	7	15	19	9	7	4	7
186	26	20	17	11	5	9	24	19	16	11	4	7
188	24	12	10	6	13	6	18	11	8	5	9	6
190	12	7	4	2	7	4	10	6	4	1	5	4
192	9	8	6	4	2	4	7	2	5	2	1	0
194	49	48	19	22	23	19	44	46	15	22	22	17
196	8	7	5	4	3	3	8	6	5	3	3	3
198	22	15	15	4	5	9	22	13	15	3	5	8
200	7	10	1	8	5	2	7	10	1	8	4	2
Total	222	222	108	108	90	90	202	202	98	98	79	79
P value	0.29		0.03		0.37		0.23		0.006		0.78	
GABRA5												
278	7	4	1	1	3	3	6	3	1	1	3	2
280	9	7	6	4	2	3	7	6	4	3	2	3
282	22	31	8	14	9	11	20	30	8	13	7	11
284	23	28	9	11	9	9	21	24	8	10	8	7
286	13	16	5	9	5	4	11	15	4	8	4	4
288	20	12	12	3	6	6	20	11	12	3	6	5
290	4	0	1	0	2	0	4	0	1	0	2	0
Total	98	98	42	42	36	36	89	89	38	38	32	32
P value	0.29		0.18		0.93		0.25		0.20		0.9	

DISCUSSION

Data presented here, gathered from a large sample of families selected for the presence of multiple alcoholics, show evidence for association of $GABA_A$ receptor gene polymorphisms and alcohol dependence. *GABRB1* provided modest but statistically significant evidence for association with alcohol dependence in the entire sample as well as in the non-Hispanic Caucasian subset (Table I). This was true for both the broader (COGA) and narrower (ICD-10) definitions of alcohol dependence. These results are consistent with the report of Parsian and Zhang [1999]. This marker also showed modest allele sharing in our genomic survey of the initial sample from the COGA study, $P < 0.05$ [Reich et al., 1998]. A different method of analysis of the initial COGA dataset using a weighted pairwise correlation method also showed evidence for an effect of a gene located near *GABRB1* [Zinn-Justin and Abel, 1999]. A genomic survey of a Southwestern American Indian population provided evidence for linkage of alcohol dependence with a marker very near the *GABRB1* locus; this was the second strongest linkage signal in the genome-wide analysis [Long et al., 1998]. This region is also linked to a quantitative phenotype related to alcoholism, the beta frequency of the human electroencephalogram, which is thought to represent an activated state of the underlying neuronal network [Porjesz et al., 2002]. The *GABRB1* tetranucleotide repeat marker studied here is located in intron 2 of the gene. Although it may not have any effect on the function of the receptor subunit, it may be in linkage disequilibrium with a functional polymorphism in the open reading frame or in a regulatory sequence. There was no evidence for linkage disequilibrium between either of the GABA-receptor genes on chromosome 5, *GABRA1*, and *GABRA6* and alcoholism. These results are consistent with most previous studies of alcohol dependence. A previous study in German subjects showed no association of sequence variants of *GABRA6*, *GABRB2*, or *GABRG2* (all in this cluster) with alcohol-dependence, familial alcoholism, or severe physiological withdrawal [Sander et al., 1999], although there was some evidence for association with antisocial alcoholism. Loh et al. [2000] studied a Japanese population and found no evidence of association of polymorphisms at the *GABRB2*, *GABRA6*, *GABRA1*, or *GABRG2* with alcoholism. They showed weak evidence ($P = 0.021$) of association of one of two polymorphisms at the *GABRG2* locus with antisocial personality. Parsian and Cloninger [1997] found no significant association of the *GABRA1* gene with alcoholism or its subtypes. Hsu et al. [1998] studied a polymorphism near the alternatively spliced exon of *GABRG2*, and found no evidence for association with alcoholism among four aboriginal groups or the Han Chinese in Taiwan. A pilot study provided preliminary evidence for association of the *GABRA6* Pro385Ser substitution and alcoholism [Schuckit et al., 1999]. However, the complete inactivation of the *Gabra6* gene in mice does not affect ethanol tolerance or withdrawal [Homanics et al., 1997, 1998].

The GABA-receptor gene cluster on chromosome 15, which is located in an imprinted area, shows interesting evidence of association with alcoholism. These genes are expressed exclusively from the paternal chromosome [Meguro et al., 1997], so the maternal gene should not influence phenotype. Consistent with this, the

association was found when analyzing transmissions of the paternal allele; the evidence was strongest for analysis of the more severe definition of alcohol dependence, ICD-10, and for the *GABRA5* gene ($P = 0.008$ in the total sample, $P = 0.004$ in the non-Hispanic Caucasian subset; Table II). There was no evidence for biased transmission of the maternal alleles, and their inclusion into the analysis greatly diluted the evidence for association (Tables II, III). Parsian and Cloninger [1997] found a weak association between *GABRA3* and alcoholism; perhaps reanalysis with parent of origin considered would strengthen the association. Noble et al. [1998] reported an association between *GABRB3* and alcoholism in an analysis that grouped the alleles into two groups on the arbitrary basis of size. There is no evidence for imprinting in the regions of chromosomes 4 and 5 that contain the other GABA-receptor gene clusters.

These analyses suggest that *GABRB1* or a very nearby gene may play a role in the risk for development of alcoholism. Our novel analysis of association of paternal (expressed) alleles provides evidence that *GABRB3* and *GABRA5*, on chromosome 15, are associated with increased risk for alcoholism. Further studies will focus on the examination of linkage disequilibrium among SNPs in each of these candidate genes.

ACKNOWLEDGMENTS

The Collaborative Study on the Genetics of Alcoholism (COGA) (H. Begleiter, SUNY HSCB, Principal Investigator: T. Reich, Washington University, Co-Principal Investigator) includes nine centers where data collection, analysis, and/or storage take place. The nine sites and Principal Investigator and Co-Investigators are: Indiana University (T.-K. Li, J. Nurnberger Jr, P.M. Conneally, H.J. Edenberg); University of Iowa (R. Crowe, S. Kuperman); University of California at San Diego (M. Schuckit); University of Connecticut (V. Hesselbrock); State University of New York, Health Sciences Center at Brooklyn (B. Porjesz, H. Begleiter); Washington University in St. Louis (T. Reich, C. R. Cloninger, J. Rice, A. Goate); Rutgers University (J. Tischfield); and Southwest Foundation (L. Almasy). This national collaborative study is supported by NIH grant U10AA08403 from the National Institute on Alcohol Abuse and Alcoholism (NIAAA). This work was also supported by AA00285 (T.F.).

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