ORIGINAL ARTICLE

Systematic Analysis of Glutamatergic Neurotransmission Genes in Alcohol Dependence and Adolescent Risky Drinking Behavior

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Context: Glutamatergic neurotransmission is implicated in alcohol-drinking behavior in animal models.

Objective: To investigate whether genetic variations in glutamatergic neurotransmission genes, which are known to alter alcohol effects in rodents, contribute to the genetic basis of alcoholism in humans.

Design: Association analysis of alcohol dependence and haplotype-tagging single nucleotide polymorphisms (SNPs) covering 10 glutamatergic genes. Resequencing of functional domains of these genes identified 204 SNPs. Haplotypes with a frequency of 5% or greater could be discriminated by 21 haplotype-tagging SNPs analyzed for association in 2 independent samples of alcohol-dependent adult patients and controls as well as adolescent trios.

Setting: Four university medical centers in the south of Germany.

Participants: One thousand three hundred thirty-seven patients and 1555 controls (study 1: 544 patients, 553 controls; study 2: 793 patients, 1002 controls). One hundred forty-four trios of 15-year-old adolescents assessed for risky drinking behavior.

Main Outcome Measures: Genotype profiles for *GLAST*; *N*-methyl-D-aspartate–receptor subunits *NR1*, *NR2A*, and *NR2B*; *MGLUR5*; *NNOS*; *PRKG2*; *CAMK4*; the regulatory subunit of *PI3K*; and *CREB* were analyzed for association with alcohol dependence using multivariate statistical analysis. Risky adolescent drinking was tested using the transmission disequilibrium test.

Results: Analysis of study 1 revealed that *NR2A* and *MGLUR5* have the greatest relevance for human alcohol dependence among the genes selected with odds ratios of 2.35 and 1.69, respectively. Replication analysis in study 2 confirmed an association of alcohol dependence with *NR2A* (odds ratio, 2.01) but showed no association with *MGLUR5*. Combined analysis of study 1 and study 2 exhibited a more significant association on the Cochran-Mantel-Haenszel test ($P < .001$) for *NR2A*; *NR2A* was associated with positive family history, early onset of alcoholism, and maximum number of drinks in adults as well as risky drinking patterns in adolescents.

Conclusion: Genetic variations in *NR2A* have the greatest relevance for human alcohol dependence among the glutamatergic genes selected for their known alteration of alcohol effects in animal models.

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ALCOHOL-RELATED DISORDERS cause 3.2% of deaths (1.8 million) and 4.0% of total disability-adjusted life-years.[1] Alcohol dependence is a complex disorder with environmental as well as genetic components and does not have a main gene effect.[2] As in other oligogenic neuropsychiatric disorders, multiple genes contribute to phenotypes of alcohol dependence. These genes involve different neurotransmitter systems, including glutamate, γ-aminobutyric acid, dopamine, opioids, serotonin, noradrenaline, and cannabinoids.[3] Unlike many neuropsychiatric disorders, alcohol dependence offers a unique research potential to elucidate the contribution of each neurotransmitter system and apply translational approaches, as a wealth of behavioral animal models exist, which allow detailed assessment of drug-related behavior.[4,5] To inform diagnostic and therapeutic developments in humans, the relevance of animal findings for human alcohol dependence and related phenotypes needs to be assessed in translational studies. Results of behavioral animal studies are often based on knockout models or pharmacological agents, which interfere to a much larger degree with signaling pathways than the usually more subtle consequences of functional genetic variations in

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frequent oligogenic disorders. For this reason and to explain a larger amount of genetic variance, we decided to analyze genes that encode functionally related proteins pertaining to one critical neurosignaling pathway, glutamatergic signaling.

Recently, a glutamatergic hypothesis has been developed to better understand the acute and chronic effects of alcohol on the brain. Alcohol affects the glutamatergic system on molecular, neurochemical, and cellular levels and this hypothesis proposes that alcohol consumption leads to enhanced glutamatergic activity in alcohol-dependent patients.[6,7] This glutamate-induced hyperexcitability is uncovered during alcohol withdrawal. Furthermore, the hypothesis suggests that augmented glutamatergic activity can contribute to craving and relapse behavior, thus providing the rationale for using antiglutamatergic compounds, such as acamprosate, for relapse prevention.[8] The role of glutamatergic neurotransmission in alcohol-drinking behavior has been analyzed using animal models and biochemical experimentation, and alterations have been identified at the presynaptic, synaptic, postsynaptic, or intracellular signaling level.[7,9] It is suggested that increases in extracellular glutamate induced by ethanol exposure may be due in part to deficits in glutamate transport.[10] Synaptic concentration of glutamate is partly regulated by glutamate aspartate transporter (GLAST). As shown in a recent animal study, decreased expression levels of GLAST result in increased synaptic glutamate concentration and increased amount of alcohol intake.[11] Glutamate receptors are primary targets of alcohol action and alcohol inhibits the postsynaptic *N*-methyl-D-aspartate (NMDA)–receptor complex,[12] thereby modulating alcohol sensitivity,[13] self-administration, relapse behavior,[7,14] and withdrawal responses.[15] Chronic alcohol exposure results in compensatory upregulation of NMDA-receptor subunits, mainly NR1, NR2A, and NR2B,[16] and can result in a hyperexcitatory state in periods of acute and conditioned alcohol withdrawal.[6,7] Pharmacological inhibition experiments show that metabotropic glutamate receptor 5 (mGluR5) modulates alcohol self-administration[17,18] and relapse behavior in rodents[19] and is a potential target for acamprosate as well.[20] Intracellularly, activation of NMDA receptors initiates a calcium-mediated signal transduction cascade activating calmodulin-dependent kinase IV and the transcription factor cyclic adenosine monophosphate–responsive element-binding protein 1 (CREB),[21] which are implicated in alcohol withdrawal[22,23] and self-administration in alcohol-preferring rats.[24] Glutamate-induced activation of CREB also occurs through a parallel pathway,[25] whereby mGluR5 and NMDA-receptor signaling converges on phosphatidyl inositol 3 kinase.[26] Phosphatidylinositol 3-kinase then activates neuronal nitric oxide synthase and guanosine monophosphate–kinase II, all of which have been implicated in regulation of alcohol sensitivity and self-administration in knockout models.[27-30]

This study is intended to address to what extent genetic variations in the glutamatergic neurotransmission genes that are shown to be correlated with alcohol drinking and relapse behavior in animal models contribute to the genetic basis of alcohol dependence in humans. In a broad resequencing analysis of each gene, we identified polymorphisms in regulatory domains, exons, and exon-intron boundaries, which constitute most functional glutamatergic variations. In a second step, all allelic information was contracted into multimarker-tagging single-nucleotide polymorphisms (SNPs), which concentrate the available functional allelic information into few predictive allelic markers.[31] These haplotype-tagging SNPs (htSNPs) were then used to perform an association study with independent replication. The first sample consisted of 544 patients with alcohol dependence and 553 controls and was used to genotype the entire set of htSNPs. Genes associated with alcohol dependence were analyzed in a second sample of 793 alcohol-dependent patients and 1002 controls (**Table 1**) and were further assessed for their role in early risky drinking behavior in a sample of 144 trios of 15-year-old adolescents and their parents.

Table 1. Alcohol-Dependent Adult Patients and Controls and Adolescent Trio Sample

Participant	No. of Participants	Age, Mean (SD), y	Sex, %	
			M	F
Study 1 Sample				
Patients	544	42.62 (9.54)	77.3	22.7
Controls	553	44.68 (13.96)	51.2	48.8
Study 2 Sample				
Patients	793	43.58 (9.93)	77.8	22.2
Controls	1002	47.96 (15.53)	59.6	40.4
Adolescent Trio Sample[a]				
Adolescents	144	14.99 (0.37)	53.1	46.9

[a] Adolescent sample consisted of 142 complete trios and 2 incomplete trios (only offspring and mother).

METHODS

PARTICIPANTS AND PSYCHIATRIC ASSESSMENT

Alcohol-Dependent Adult Patients and Controls

For study 1, 544 patients were recruited from the psychiatry and addiction medicine departments at the university hospitals in Munich, Mainz, and Mannheim, Germany. Table 1 presents the characteristics of our sample. All patients were consecutively admitted for inpatient addiction treatment and fulfilled *DSM-IV* criteria for alcohol dependence. All participants were of German descent. In Munich and Mannheim, patients were assessed using the Semi-Structured Assessment for the Genetics of Alcoholism[32] and the Structured Clinical Interview for *DSM-III-R*, respectively. In Mainz, the Composite International Diagnostic Interview[33] and the Michigan Alcoholism Screening Test were used. Interviews were performed by trained staff members and rated independently. Genetically enriched phenotypes across study 1 were early onset, indicating onset of alcohol dependence before age 25 years. Positive family history for alcoholism included first- or second-degree relatives. One drink was defined as 17 g of alcohol.

Five hundred fifty-three controls were recruited in Munich, Mainz, and Mannheim (Table1). Munich controls were randomly selected from city registration. Controls from Mainz

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and Mannheim were from hospital personnel and board advertisement. A detailed medical and psychiatric history was performed and all Axis 1 psychiatric diagnoses were excluded.

For study 2, 793 patients were recruited from the Department of Psychiatry at Regensburg University (Table 1). All patients were admitted consecutively for inpatient treatment and met criteria of alcohol dependence according to *DSM-IV*. All participants were of German descent. Parents of the participants were living in Bavaria and participants themselves were born and raised in this area. Written informed consent was obtained from all participants before the investigation.

Diagnosis was assessed after alcohol withdrawal by the Composite International Diagnostic Interview[33] performed by trained staff who rated participants independently. Patients with a lifetime history of schizophrenia or an addiction to drugs other than alcohol or tobacco were excluded. Genetically enriched phenotypes across sample 2 were defined similar to sample 1.

One thousand two individuals from Bonn were recruited from 2001 to 2003 within the German National Research Project to serve as controls for genetic studies in several neuropsychiatric phenotypes. Population-based recruitment was performed in collaboration with the local census bureau. Participants were screened for neurological and psychiatric disorders with self-report questionnaires adapted from the German version of the Inventory to Diagnose Depression[34,35]; smoking and drinking was screened with the Fagerström Tolerance Questionnaire and the Alcohol Use Disorders Identification Test, respectively.[36,37] More than 96% of the participants were of German or Western European origin as ascertained by place of birth of their grandparents. For all patients and controls, written informed consent was obtained before study participation.

Adolescent Trio Sample

One hundred forty-two complete trios and 2 incomplete trios were recruited from the Mannheim Study of Risk Children, a cohort of first-born children (Table 1).[38] Alcohol consumption in the last 6 months before assessment was evaluated at age 15 years using the Lifetime Drinking History interview.[39] Lifetime prevalence of being drunk was defined as the participant never having been drunk vs having been drunk. High/low maximum amount of alcohol intake on 1 occasion was established using a median split at 2 or more standard alcoholic drinks. All participants were of Central European descent. Written informed consent was obtained from all individuals when they were in a state of full legal capacity. The study was approved by the ethics committees of the Landesärztekammer Rheinland-Pfalz and the universities of Regensburg, Heidelberg, and Munich.

MUTATION SCREENING AND IDENTIFICATION OF htSNPs

Identification of SNPs was performed by sequencing 32 DNA samples from white individuals. Sixteen DNA pools consisting of an equimolar mixture of 2 DNA samples were prepared and used as polymerase chain reaction templates. For each gene, primers were chosen to amplify the regulatory domains and the exon-containing DNA fragments, including exon-intron boundaries. Polymerase chain reaction was performed in a 15-μL reaction mixture containing 25 ng of DNA (the list of the primers for each gene is available at http://www.cng.fr). Sequencing reactions were performed using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, California). Alignment of experimental results and identification of SNPs were performed using the Genalys software developed by the Centre National de Génotypage. Haplotype-tagging SNPs were selected to discriminate haplotypes with a frequency of 5% or greater.

GENOTYPE ANALYSIS

Participants' DNA was prepared from whole blood with standard salting out methods. Single nucleotide polymorphisms were genotyped using the TaqMan system at the Centre National de Génotypage, University of Kiel Institute of Psychiatry. Probes and primers were from the Assay-by-Design system (Applied Biosystems). Polymerase chain reactions were performed in Biometra T1 thermocyclers (Biometra, Goettingen, Germany), and fluorescence results were determined by using an ABI Prism 7900HT sequence detector end-point read (Applied Biosystems). Process and genotyping data were exported into an internal laboratory information management system. In study 1, complete genotypes were obtained from 823 individuals, including 410 patients and 413 controls, which were included in the statistical analysis. In the follow-up study, 1795 individuals, including 793 patients and 1002 controls, were genotyped using 2 independent genotyping techniques, our in-house TaqMan and commercial genotyping (PreventionGenetics, Marshfield, Wisconsin) with 100% concordance.

STATISTICAL ANALYSIS

In study 1, multivariate statistical analysis for genotype × phenotype association was performed using a logistic regression approach. All htSNPs were in Hardy-Weinberg equilibrium (data not shown).[40] Alternative haplotype analyses were done with COCAPHASE 2.404.[41] In study 2, multilocus genotypes identified in study 1 were analyzed for replication. Trio analyses were performed using the transmission disequilibrium test. Transmission disequilibrium test was performed using tdtphase, version 2.404, which is part of the UNPHASED software package.[42]

GENEWISE LOGISTIC REGRESSION ANALYSIS

For logistic regression analysis, 4 htSNPs with a high correlation with other htSNPs ($r > 0.7$) were eliminated. The remaining htSNPs were coded into 10 categorical variables representing the 10 genes. Dummy variables were created, each representing 1 multilocus genotype within 1 gene. Rare genotypes (< 5% of the sample number) were grouped together. The most frequent genotype was taken as the reference group. These dummy variables were included as predictors in the regression model. Testing the relevance of these genes for prediction of alcohol dependence was performed by testing the null hypothesis that none of the analyzed genotypes within each gene differ from the reference group. The genes were ranked based on obtained *P* values (**Table 2**). Using regression coefficients, multilocus genotypes were identified for *NR2A* (OMIM *138253) and *MGLUR5* (OMIM *604102), which predict a particular high (or low) risk for alcohol dependence compared with the reference group (**Table 3**). Taking sex into account led to the similar results for relevance of genes as well as for detecting risk groups within genes (data not shown). These extreme groups were compared to create an effect measure for the amount of the gene's influence on status and phenotypes.

The extreme group analysis was carried out for the replication study. The Fisher exact test (2-sided) was used to evaluate the association between extreme groups for alcohol dependence. Evidence of replication, rather than multiple testing corrections, was used to evaluate the significance of associated genes. To comprehensively assess the reproducible results, we conducted the Cochran-Mantel-Haenszel test[43] on the combined data set, which includes samples from original and replication case-control studies. Pooled odds ratios (ORs) with 95% confidence intervals (CIs) and *P* values were obtained from Cochran-Mantel-Haenszel statistics (**Figure 1**).

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Table 2. Genewise Multiple Logistic Regression of Alcohol Dependence[a]

Gene	Wald χ^2	*df*	*P* Value[b]
MGLUR5	9.9908	5	.08
NR2A	9.7770	5	.08
PIK3R1	9.5969	6	.14
CAMK4	10.1689	7	.18
PRKG2	2.6170	2	.27
GLAST	4.9746	5	.42
NOS1	1.0049	2	.61
CREB1	0.3591	2	.84
NR1	0.1980	2	.91
NR2B	0.1593	2	.92

[a] Genotypes of all markers were within Hardy-Weinberg equilibrium.
[b] Results from testing the null hypothesis (ie, none of the multilocus genotypic groups within a gene differ from reference group).

Table 3. Genewise Identification of Risk vs Protective Multilocus Genotypes[a]

Genotype	Odds Ratio (95% Confidence Interval)
***NR2A* rs9924016-rs2072450**	
DelIns-CC	1 [Reference]
DelDel-CC[b]	1.234 (0.877-1.735)
DelDel-AC	1.066 (0.679-1.672)
DelIns-AC[c]	0.503 (0.272-0.933)
InsIns-CC	0.926 (0.511-1.676)
All other	1.787 (0.595-5.366)
***MGLUR5* rs3462-rs3824927**	
GG-CA	1 [Reference]
GG-AA	0.834 (0.572-1.218)
GG-CC[b]	1.252 (0.798-1.965)
GA-CA[c]	0.723 (0.468-1.119)
GA-CC	1.151 (0.701-1.891)
All other	3.895 (1.010-15.018)

[a] Odds ratio estimates for *NR2A* and *MGLUR5* genotypes taken from full logistic regression model comprising all 17 markers of all 10 genes. Reference group is most frequent multilocus genotype of each gene. Small categories (groups that comprise < 5% of whole sample for a particular genotype) are pooled into 1 category and excluded from risk group selection.
[b] Risk group for alcohol dependence.
[c] Protective group.

RESULTS

IDENTIFICATION AND SELECTION OF htSNPS AND POWER CALCULATION

Based on behavioral animal experimentation and pharmacological data (**Table 4**), we selected the genes *GLAST*; NMDA-receptor subunits *NR1*, *NR2A*, and *NR2B*; *MGLUR5*; *NNOS*; *PRKG2*; *CAMK4*; the regulatory subunit of *PI3K*; and *CREB* (**Figure 2**). Identification of variations in the genes selected was performed by sequencing of regulatory domains, exons, and exon-intron boundaries (see "Methods" section) (**Table 5**). Two hundred four genetic variations were identified (http://www.cng.fr). Fifty-four polymorphisms had no reference SNP identifier at the time of discovery. To date, 4 additional SNPs have been given reference SNP identifiers (Table 5). Previously unknown polymorphisms added information to the haplotypes (frequency ≥5%) for the genes *CAMK4, PIK3R1, PRKG2*, and *SLC1A3*. Except for SNP *SLC1A3*-95103, which was genotyped in the present study, all other polymorphisms that contribute to haplotypes with a frequency of 5% or greater were tagged by multimarker combinations of other known variants (Table 5). The haplotypic structure (frequency ≥5%) of the analyzed genes was not diversified by the newly discovered variants (except in *SLC1A3*).

As a first step toward the identification of htSNPs, SNPs with a minor allele frequency of less than 5% were discarded. If groups of SNPs existed in which scored genotypes were identical for all samples, then a single SNP from the group was selected. The selected SNP was that with the least missing data or simply the first in the event of a tie. Haplotypes for the remaining SNPs were then estimated using the expectation-maximization algorithm, and a subset of htSNPs was selected so that all haplotypes with a frequency of 5% or greater could be distinguished. This process resulted in selection of 21 htSNPs (Table 4), which were genotyped in study 1. Genes shown to be associated with alcohol dependence were analyzed for replication in a second, independent sample. Disease severity, as defined by the mean of the number of *DSM-IV* symptoms in sample 1, was 5.74 compared with sample 2 with a mean of 6.07 (analysis of variance, $F = 18.184$; $P < .001$) (**Table 6**). Power calculation of the combined data set comprising 1555 controls and 1337 cases in an additive disease model revealed 98% power to detect a genotype relative risk of 1.25.

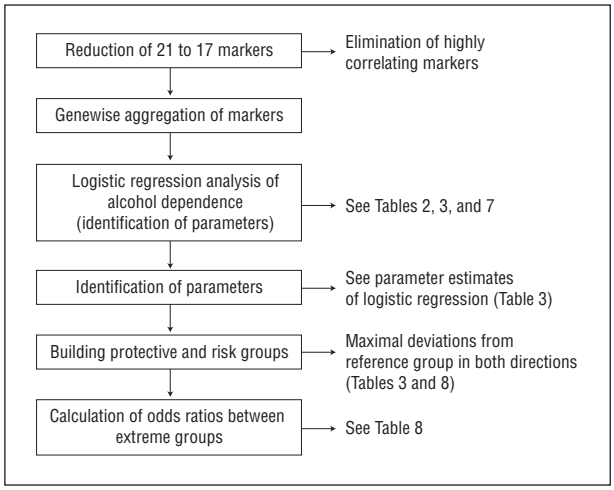

Figure 1. Statistical modeling overview.

IDENTIFICATION OF RISK GENOTYPES FOR ALCOHOL DEPENDENCE

Using a genewise, multiple regression approach, variations in 2 genes, *NR2A* and *MGLUR5*, were shown to be the most powerful predictors of alcohol dependence (Table 2). Detailed haplotype frequencies and haplotypic structures for *GRIN2A* and *GRM5* are presented in **Table 7**. In these 2 genes, newly discovered variants added no ad-

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Table 4. Selection of Genes and htSNP Identification

Full Gene Name	Gene Symbol	Chromosome	No. of SNPs Detected	htSNPs (CNG SNP Identifier[a])	Behavioral Animal Studies
Calcium/calmodulin-dependent protein kinase IV	*CAMK4*	5q21-23	17	rs387238 rs3797739 rs25917 22222019 rs3797740 rs3733995	Analgesic tolerance (opioid)[44]
cAMP responsive element binding protein 1	*CREB1*	2q32	8	rs2551920	Alcohol self-administration[24]
Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1	*NR1/GRIN1*	9q34	5	22221997 (22222216)	Alcohol-associated anxiolysis and motor impairment[32]
Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2A	*NR2A/GRIN2A*	16p13	11	rs6497540 rs9924016 rs11642764 rs2072450	Alcohol sensitivity,[10] alcohol self-administration[33]
Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2B	*NR2B/GRIN2B*	12p12	29	rs2072539 or (rs890) (rs1806195)	Alcohol withdrawal[12]
Glutamate receptor, metabotropic 5	*GRM5*	11q14	19	rs3462 rs3824927	Alcohol self-administration,[17,18] alcohol seeking/reinforcement, relapse[19]
Nitric oxide synthase 1 (neuronal)	*NOS1*	12q24	41	rs1875140 rs3741476	Alcohol self-administration, sensitivity[27]
Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	*PIK3R1*	5q13	22	rs706713 rs2302975 rs171649	Alcohol sensitivity,[29] sensitization (cocaine)[45]
Protein kinase, cGMP-dependent, type II	*PRKG2*	4q13	10	rs9992933	Alcohol self-administration, sensitivity[28]
Solute carrier family 1 (glial high affinity glutamate transporter), member 3	*GLAST/SLC1A3*	5p13	19	(22221995) rs10057629	Alcohol self-administration[8]

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; CNG, Centre National de Génotypage; htSNP, haplotype-tagging single-nucleotide polymorphism; SNP, single-nucleotide polymorphism.
[a]Identifier for SNPs not present in the Single-Nucleotide Polymorphism Database.

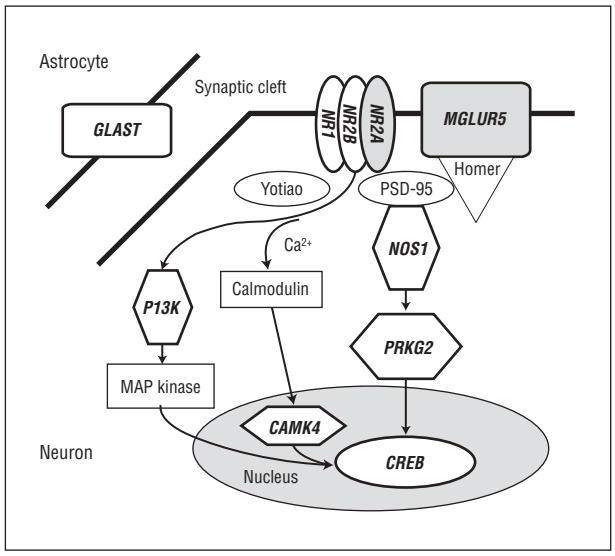

Figure 2. Glutamatergic neurotransmission (candidate genes selected for analysis are indicated by a bold frame). MAP, mitogen-activated protein; PSD-95, postsynaptic density-95.

ditional information to haplotypes with a frequency of 5% or greater. Each of these genes was characterized by 2 htSNPs (the remaining 2 htSNPs of *NR2A* were eliminated because they were correlated [$r > 0.7$] to the htSNPs analyzed). In the case of *NR2A*, these variations were a C-to-A substitution in rs2072450 (intron 11) and a deletion/insertion in rs9924016 (intron 7). In the case of *MGLUR5*, these variations were the C-to-A exchange in rs3824927 (3′ flanking sequence) and the G-to-A exchange in rs3462 (exon 8) (**Figure 3** and **Figure 4**). To assess the effect size of the genetic variations on alcohol dependence, protective and risk genotypes were identified based on their regression coefficients (Table 3). This analysis showed that carriers of the *NR2A* risk genotypes rs2072450 CC and rs9924016 Del/Del had an OR of 2.35 (95% CI, 1.37-4.03) of developing alcohol dependence compared with carriers of the protective genotypes rs2072450 AC and rs9924016 Del/Ins (Fisher exact test, $P = .002$) (Table 3). In the case of *MGLUR5*, carriers of the risk genotypes rs3824927 C/C and rs3462 G/G had an OR of 1.69 (95% CI, 1.06-2.69) of developing alcohol dependence compared with carriers of the protective genotypes rs3824927 CA and rs3462 GA ($P = .03$) (**Table 8**). In our samples, the protective genotype was heterozygous for all *NR2A* and *MGLUR5* polymorphisms because no individuals were observed who carried a homozygous protective genotype. Haplotype analysis of *NR2A* and *MGLUR5* genotypes did not yield an increase in OR compared with results of the regression analysis (data not shown). Correlation analysis revealed no evidence for confounding association between *NR2A* and *MGLUR5* markers and age or sex (data not shown).

In the replication, we performed a confirmatory independent analysis of the combined *NR2A* and *MGLUR5* genotypes in a sample of 793 alcohol-dependent patients and

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Table 5. List of Polymorphisms Discovered by Resequencing

mRNA	CNG Discovery Identifier[a] (rs Identifier[b])	Location	Polymorphism Flanking Sequences	Allele, Reference/ Variant	Frequency	Haplotype	Marker[c]
			CAMK4				
NT_034772.5	22222024 (rs34578342)	Intron 1	AAAAAAA**N**TACTCTT	AA/--	0.850	+	rs387238, rs3797739, rs25917
NM_001744.3	22222019	Intron 2	CTGACAA**N**TTGTGAT	G/C	0.094	–	
	22222022	Intron 2	ATGTTAC**N**TCAGAAG	G/A	0.017	–	
	22222025 (rs34007984)	Intron 6	GAAGTTT**N**TGCTCTT	T/G	0.339	+	rs387238, rs3797739, rs25917
	22222020	Intron 8	ATGAACA**N**TGCTTTT	T/C	0.019	–	
	22222021	Intron 9	TTTTACC**N**ATATTAA	C/-	0.016	–	
	22222023	Intron 9	CCATATT**N**TGCCATA	AA/--	0.016	–	
			CREB				
NT_005403.15	22222171	Intron 1	GAAACAG**N**CAAGGGA	C/T	0.017	–	
NM_004379.2	22222172	Exon 2	TGAAAAC**N**AACAAAT	C/G	0.016	–	
	22222173	Intron 5	CAAGTGT**N**TAAACCT	A/G	0.016	–	
	22222168	Exon 6	CATTAAC**N**ATGACCA	C/G	0.016	–	
	22222169	Exon 8	CCCTCCT**N**AAGAAGT	C/T	0.016	–	
	22222170	Exon 8	GCACAAG**N**AAAGTTA	G/A	0.016	–	
			GRIN1				
NT_024000.16	22221999	Exon 1	GGGGGCG**N**TGGGAGC	C/T	0.000	–	
NM_000832.4	22221997	Intron 2	CAATACA**N**ACTCTCT	C/T	0.016	–	
	2221998	Intron 8	CACCCCC**N**TCTGGCC	-/C	0.000	–	
			GRIN2A				
NT_010393.15	22222082	Intron 1	CCCACCT**N**CCCCCTT	G/-	1.000	–	
NM_000833.2	22222084	Exon 4	CGTAGGA**N**ACAGAAA	C/G	0.016	–	
	22222076	Intron 6	TTTTAAA**N**TGATTAT	A/T	0.078	–	
	22222077	Intron 6	ATTAAAC**N**GGTAAAA	T/-	0.047	–	
	22222081 (rs35524162)	Intron 10	AGAGTAG**N**AAAAGAA	C/T	0.017	–	
			GRIN2B				
NT_009714.16	22222065 (rs34454922)	Intron 1	ATAACCA**N**ATTATAA	AAT/- --	0.125	–	
NM_000834.2	22222060	Intron 2	TCAACAT**N**CCTTAGT	G/A	0.047	–	
	22222061	Intron 2	TATACCT**N**TTTGAAG	T/C	0.016	–	
	22222070	Intron 8	AACCTAC**N**AATACAC	T/-	0.063	–	
	22222068	Exon 13	TCCAGGA**N**CCCATAA	A/C	0.016	–	
			GRM5				
NT_008984.17	22222154	Exon 1	TAGCTAC**N**AACAAGC	C/G	0.120	–	
NM_000842.1	22222156	Exon 1	GAAAATT**N**AGGAGGG	G/C	0.021	–	
	22222161	Intron 2	TTAAGCT**N**TATTTCC	G/C	0.016	–	
	22222165	Intron 4	CTACTCT**N**CAGAGGA	C/G	0.018	–	
	22222153	Intron 5	TAGATTC**N**ATTTTAC	G/A	0.016	–	
	22222157	Intron 6	CCCAGAG**N**TCTGAAG	C/G	0.016	–	
	22222160	Exon 7	TCGAAGA**N**ACTGTAC	A/G	0.016	–	
	22222163	Exon 7	AGATCTT**N**TTCTTGC	G/A	0.016	–	
	22222166	Intron 7	TGAGGAA**N**GGCCGTG	G/A	0.016	–	
			NOS1				
NT_009775	17444822	Intron 21	TGGGCCA**N**GGTTGAT	T/G	0.017	–	
NM_000620.1							
			PIK3R1				
NT_006713.14	22222183	5′-flanking	GACTTCC**N**AGAATAT	A/G	0.016	–	
NM_181504.2	22222184	5′-flanking	CAAATCA**N**GTACAGT	T/C	0.016	–	
	22222185	5′-flanking	ATGTTGG**N**CATCATC	T/C	0.016	–	
	22222190	5′-flanking	ATTGTTA**N**CCTATTG	A/C	0.150	–	rs706713
	22222192	5′-flanking	GCTTTTC**N**GATTCTC	A/G	0.017	–	
	22222186	Exon 10	GATGAAG**N**GCTTACT	C/T	0.016	–	
	22222187	Exon 10	TCTCTGC**N**TGCAGGG	G/T	0.016	–	
	22222189	Exon 10	TTCCCCA**N**CCCAGTT	C/T	0.016	–	
	22222191	Exon 10	GCTGTTA**N**CCCAAGG	G/A	0.016	–	
			PRKG2				
NT_016354.17	C22222049	Intron 2	ATGAGAA**N**CATAAAA	T/C	0.016	–	
NM_006259.1	C22222046	Intron 5	CGGTGCG**N**GCACCCA	C/T	0.156	+	rs9992933
	C22222047	Intron 6	GCTTTGC**N**TTGCAAC	A/G	0.016	–	
			SLC1A3				
NT_006576.15	22221992	Intron 1	AACTCAT**N**TCTCTTT	G/T	0.019	–	
NM_004172.3	22221990	Exon 4	CGCTAGA**N**AGTAAGG	T/C	0.017	–	
	22221991	Intron 8	AGTGAGA**N**ACTGTTT	T/C	0.619	+	CNG 95103, rs10057629
	22221995	Intron 8	TTTGCAG**N**GTATATG	C/T	0.555	+	genotyped
	22221996	Intron 8	CTACGTG**N**GGAGCTT	G/C	0.125	+	CNG 95103, rs10057629
	22221993	Exon 10	AAGAATG**N**ACTTAAT	G/C	0.055	–	

Abbreviations: CNG, Centre National de Génotypage; mRNA, messenger RNA; rs, reference single-nucleotide polymorphism; SNP, single-nucleotide polymorphism; +, does contribute to haplotype frequency of 5% or greater; –, does not contribute to haplotype frequency of 5% or greater.

[a] Centre National de Génotypage discovery identifiers of newly discovered SNPs (no rs identifier at the time of resequencing).

[b] Reference SNP identifier (Single-Nucleotide Polymorphism Database, build 126) assigned to SNPs discovered after resequencing project.

[c] Markers with rs numbers that multimarker tag the SNPs discovered during resequencing.

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Table 6. Comparison of Severity of Alcohol Dependence in Study 1 vs Study 2[a]

No. of *DSM-IV* Symptoms	No. of Patients (%)	
	Sample 1 (N=345)	Sample 2 (N=793)
3	27 (7.83)	36 (4.5)
4	43 (12.46)	58 (11.9)
5	66 (19.13)	129 (16.3)
6	65 (18.84)	155 (19.5)
7	144 (41.74)	415 (52.3)

[a]Mean number of symptoms was 5.742 in study 1 and 6.078 in study 2 ($P<.001$, analysis of variance).

1002 controls. We found that carriers of the *NR2A* risk genotypes rs2072450 CC and rs9924016 Del/Del had an OR of 2.01 (95% CI, 1.15-3.50) of developing alcohol dependence compared with carriers of the protective genotypes rs2072450 AC and rs9924016 Del/Ins ($P=.02$) (Table 8). However, analysis of the *MGLUR5* risk genotypes did not yield significant results ($P=.62$) (Table 8).

For joint analysis of studies 1 and 2, the contingency tables (2×2) with risk and protective genotypes were combined for *NR2A* and *MGLUR5*. This analysis revealed that for alcohol dependence, the *NR2A* risk genotypes rs2072450 CC and rs9924016 Del/Del had a pooled OR of 2.18 (95% CI, 1.48-3.21) compared with the protective genotypes rs2072450 AC and rs9924016 Del/Ins ($P<.001$) (Table 8). No evidence was found of an association of alcohol dependence with the *MGLUR5* risk genotype vs protective genotype ($P=.38$) (Table 8). Using the Cochran-Armitage trend test, we obtained similar results for *NR2A* and *MGLUR5* and their protective vs intermediate vs risk genotypes (data not shown). Correlation analysis revealed no evidence for a confounding association between the *NR2A* markers and age or sex (data not shown).

ASSOCIATION ANALYSIS OF *NR2A* GENOTYPES IN PHENOTYPES CARRYING A HIGH GENETIC LOAD

Patients with a positive family history and those with early onset of the disorder are thought to carry a high genetic load for alcohol dependence.[3] Amount of alcohol intake measured as maximum number of drinks has been shown to be genetically influenced.[46] We selected these phenotypes for further exploratory analysis of *NR2A* risk genotypes. We analyzed the association of these phenotypes and severity of the dependence as defined by the number of *DSM-IV* symptoms and found an association with amount of drinking in both samples (study 1: Spearman correlation coefficient, 0.167; $P=.003$; study 2: correlation coefficient, 0.255; $P<.001$) and positive family history in sample 2 ($F=5.789$, $P=.02$, analysis of variance) but not in other phenotypes (data not shown). Positive family history was associated with *NR2A* risk vs protective genotype in study 1 ($P=.001$) and study 2 (OR, 2.37; 95% CI, 1.29-4.36; $P=.007$), with joint analysis of both samples revealing an OR of 2.68 (95% CI, 1.58-4.54; $P<.001$) (**Table 9**). Early onset of alcohol dependence was associated with *NR2A* risk vs protective genotypes in study 1 (OR, 3.54; 95% CI, 1.22-10.3; $P=.01$) and study 2 (OR, 2.67; 95% CI, 1.42-5.02; $P=.002$), with joint analysis revealing an OR of 3.69 (95% CI, 2.0-6.81; $P<.001$) (Table 9). While no association of maximum number of drinks with *NR2A* risk vs protective genotypes was observed in study 1 ($P=.35$), study 2 showed an association (OR, 3.21; 95% CI, 1.52-6.76; $P=.002$), with joint analysis revealing an OR of 2.70 (95% CI, 1.45-5.03; $P=.001$) (Table 9). The joint Cochran-Armitage trend test produced similar results for association between *NR2A* genotypes and the phenotypes in question (data not shown).

RISKY DRINKING BEHAVIOR IN ADOLESCENTS

Since we found an association between *NR2A* genotypes and positive family history, early onset of alcohol dependence, and maximum number of drinks, we were interested to know if the genetic variations identified in alcohol-dependent adult patients may constitute a risk factor for risky drinking in adolescents. We used a transmission disequilibrium test in a sample of 144 trios composed of 15-year-old adolescents and their parents (see the "Methods" section). Our results show an overtransmission of the C allele of the rs2072450 polymorphism in *NR2A* in both lifetime prevalence of drunkenness ($P=.04$) and maximum amount of alcohol intake/occasion, a measure for heavy drinking ($P=.02$) (**Table 10**).

COMMENT

We show that genetic variations in *NR2A* have the highest relevance for human alcohol dependence among the glutamatergic neurosignaling genes selected for their known alteration of alcohol effects in animal models. Our analysis is based on a broad resequencing approach of 10 genes, which led to the identification of 204 SNPs, 50 of which are not listed in the National Center for Biotechnology Information (NCBI) Reference Sequence database. Haplotype analysis revealed that the most frequent haplotypes (>5%) could be discriminated by 21 htSNPs, which serve to decrease redundancy of SNP information and represent the most parsimonious set to describe the sum of genetic variations of functionally relevant domains of our candidate genes. Because the *DSM-IV* diagnosis of alcohol dependence contains a number of genetically heterogenous phenotypes resulting in limited effect sizes of each individual gene, we were interested in maximizing sensitivity of our analysis by using a statistical model comparing combined risk with protective genotypes. To minimize the danger of false-positive associations, we performed an independent replication of positive results in a combined sample, which carried 98% power to detect even small genetic effects (genotype risk ratio, 1.25).

Our approach establishes the relevance of *NR2A* for alcohol dependence, as indicated by significant associations and comparable ORs of risk vs a protective genotype of 2.35 and 1.83 in the first and second samples, respectively, which result in a joint OR of 2.18. Analysis of phenotypes enriched for genetic loading increased the OR in the case of positive family history to 2.68, in

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Table 7. Haplotypes of *GRIN2A* and *GRM5*[a]

			GRIN2A			
Haplotype frequency	0.3567	0.1782	0.1250	0.1064	0.0894	0.0349
Haplotype						
CNG 552	1[b]	1[b]	1[b]	1[b]	1[b]	1
CNG 245672	0[b]	0[b]	0[b]	0[b]	0[b]	0
rs8049651	0	1	1	0	0	1
CNG 334187	0[b]	0[b]	0[b]	0[b]	0[b]	0
CNG 342380	0[b]	0[b]	0[b]	0[b]	0[b]	0
rs6497540	0	1	1	1	0	1
rs9924016	0	0	1	0	0	0
rs11642764	0	1	1	0	0	0
rs35524162	0[b]	0[b]	0[b]	0[b]	0[b]	0
rs9806806	0	1	1	0	0	0
rs2072450	0	0	0	0	1	0
			GRM5			
Haplotype frequency	0.3451	0.3257	0.0508	0.030		
Haplotype						
CNG 5420	0[b]	0[b]	0[b]	0		
CNG 5441	0[b]	0[b]	0[b]	0		
rs10741300	1	1	1	1		
CNG 203598	0[b]	0[b]	0[b]	0		
rs17770948	0	0	0	0		
rs2306153	0	0	0	0		
CNG 448806	0[b]	0[b]	0[b]	0		
rs2306154	0	0	0	0		
CNG 456324	0[b]	0[b]	0[b]	0		
rs2292102	0	0	0	0		
CNG 463075	0[b]	0[b]	0[b]	0		
rs10831110	0	0	0	0		
CNG 485533	0[b]	0[b]	0[b]	0		
CNG 485852	0[b]	0[b]	0[b]	0		
rs4753486	0	1	0	1		
CNG 528025	0[b]	0[b]	0[b]	0		
rs566277	0	0	0	0		
rs3462	1	0	0	1		
rs3824927	0	1	0	1		

Abbreviation: CNG, Centre National de Génotypage.
[a] Only common haplotypes (≥ 5% frequency) were considered for this study.
[b] Markers that were newly discovered during resequencing do not contribute to the haplotypes of a frequency of 5% or greater in these 2 genes.

the case of early onset to 3.69, and in the case of maximum number of drinks to 2.70. Our finding of an association of *NR2A* genotypes with phenotypes of risky drinking, including prevalence of drunkenness and a measure for heavy drinking patterns in 144 adolescent trios, suggests a role for *NR2A* in early stages of alcohol use and indicates its relevance for developmental trajectories of alcohol use disorders. A number of prospective and retrospective studies have shown that early-adolescent alcohol use is significantly associated with alcohol-related problems and disorders in adulthood.[47,48]

Genetic variants in public databases are not an exhaustive collection of existing human variation. Resequencing of regions with particular biological importance can complete this collection. However, despite the fact that resequencing the major functional portions of the glutamatergic genes identified 50 SNPs that are not listed in the NCBI Reference Sequence database, this exercise contributed only limited information compared with the frequent haplotypic structures revealed solely by NCBI Reference SNP polymorphisms.

Alcohol has been shown to regulate expression of *NR2A* in brain regions relevant for addiction-related neurobiological processes, including amygdala and hippocampus.[14,49,50] The NR2A subunits are among the NMDA-receptor subunits that are most sensitive to the inhibitory effects of ethanol.[51] A recent study in healthy individuals with a familial vulnerability to alcoholism showed an attenuated response to ketamine, which acts partially as an NR2A antagonist[52] to perceptual alterations and dysphoric mood in those individuals with a positive family history of alcohol dependence, suggesting a contribution of NR2A to subjective responses to alcohol.[53] Animal experimentation indicates 2 behavioral mechanisms through which NR2A may contribute to alcohol-drinking behavior and alcohol dependence; *Nr2a* knockout mice failed to show evidence for conditioned place preference, suggesting an impairment in learned reward-related responses to ethanol.[49] An alternative hypothesis rests on the finding that *Nr2a* knockout mice exhibit decreased anxiety-like behavior across multiple tests,[50] suggesting stress-induced gene × environment interactions resulting in increased

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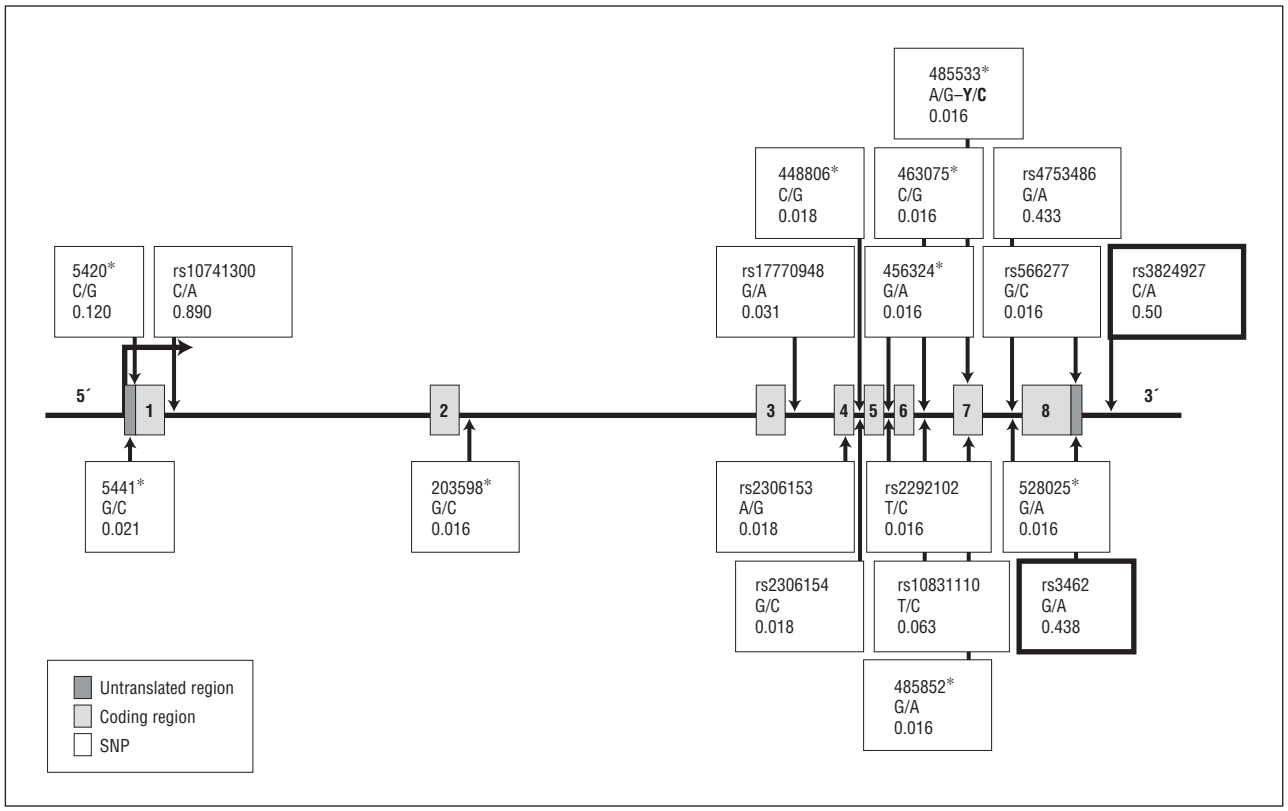

Figure 3. Schematic representation of the genomic organization of *MGLUR5*. Tagging single-nucleotide polymorphisms (SNPs) are indicated by bold frames. Introns are indicated by horizontal lines connecting the boxes. Localizations of the polymorphisms (arrows, not drawn to scale), nomenclature of the SNPs, nucleotide alleles, and base frequencies. Nomenclature according the Single-Nucleotide Polymorphism Database (reference SNP identification numbers). * Indicates SNP discovery numbers of the Centre National de Génotypage.

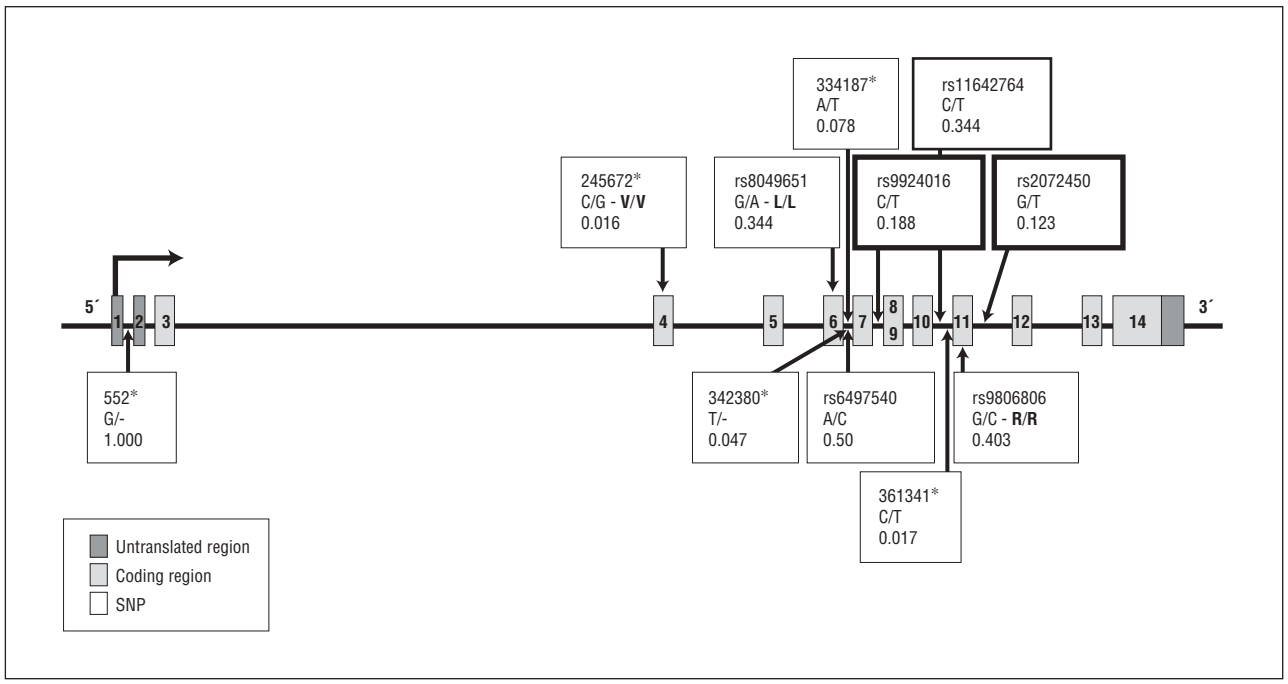

Figure 4. Schematic representation of the genomic organization of *NR2A* (exons 8 and 9 are fused into 1 rectangle). Tagging single-nucleotide polymorphisms (SNPs) are indicated by bold frames. Introns are indicated by horizontal lines connecting the boxes. Localizations of the polymorphisms (arrows, not drawn to scale), nomenclature of the SNPs, nucleotide alleles, and base frequencies. Nomenclature according to the Single-Nucleotide Polymorphism Database (reference SNP identification numbers). * Indicates SNP discovery numbers of the Centre National de Génotypage.

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Table 8. Identification and Analysis of Extreme Genotypes for Alcohol Dependence in Study 1 and Study 2

			Study 1				Study 2				Total	
Gene	Genotype 1	Genotype 2	Affected Individuals, No.	Controls, No.	OR (95% CI)	*P* Value[a]	Affected Individuals, No.	Controls, No.	OR (95% CI)	*P* Value[a]	OR (95% CI)	*P* Value[b]
NR2A	rs2072450	rs9924016										
Risk	CC	Del/Del	202	172	2.35 (1.37-4.03)	.002	60	58	2.01 (1.15-3.50)	.02	2.18 (1.48-3.21)	< .001
Protective	AC	Del/Ins	23	46			32	62				
MGLUR5	rs3462	rs3824927										
Risk	GG	CC	83	61	1.69 (1.06-2.69)	.03	118	155	0.92 (0.66-1.27)	.62	1.13 (0.86-1.47)	.38
Protective	GA	CA	66	82			137	165				

Abbreviations: CI, confidence interval; OR, odds ratio.
[a] Fisher exact test.
[b] Cochran-Mantel-Haenszel test.

Table 9. Association Analysis and Replication of *NR2A* With Genetically Enriched Phenotypes of Alcohol Dependence

	Study 1				Study 2				Total	
Measure	Affected Individuals, No.	Controls, No.	OR (95% CI)	*P* Value[a]	Affected Individuals, No.	Controls, No.	OR (95% CI)	*P* Value[a]	OR (95% CI)	*P* Value[b]
Age at onset < 25 y										
Risk	34	172		.001[c]	50	58	2.67 (1.42-5.02)	.002	3.69 (2.0-6.81)	< .001
Protective	0	46			20	62				
Positive family history										
Risk	53	172	3.54 (1.22-10.3)	.01	51	58	2.37 (1.29-4.36)	.007	2.68 (1.58-4.54)	< .001
Protective	4	46			23	62				
Maximum No. of drinks										
Risk	29	172		.35	36	58	3.21 (1.52-6.76)	.002	2.70 (1.45-5.03)	.001
Protective	4	46			12	62				

Abbreviations: CI, confidence interval; OR, odds ratio.
[a] Fisher exact test.
[b] Cochran-Mantel-Haenszel test.
[c] Calculation of OR is not possible owing to empty cell in contingency table.

Table 10. Analysis of the Role of *NR2A* in Initiation and Risky Drinking in Adolescent Trios

Measure	Not Randomly Transmitted Allele/Haplotype	T:U Ratio	Total LRS	*P* Value
Lifetime prevalence drunkenness				
rs2072450	C	17:7	4.296	.04
rs11642764	A	24:20	0.364	.55
Maximal amount of drinking/occasion (median split)				
rs2072450	C	23:10	5.263	.02
rs11642764	A	29:19	2.099	.15

Abbreviations: LRS, likelihood ratio statistic; T:U, transmitted to untransmitted.

alcohol drinking. While our samples were not suited for confirmatory gene × environment analyses, exploratory findings in the adolescent trios suggest that association of an *NR2A* genotype with amount of drinking is only present in those adolescents with high psychosocial stress but not in those with low psychosocial stress (data not shown). This indication clearly requires formal analysis in a larger sample set.

While this study is the first to find evidence of an association of *NR2A* with alcohol dependence and alcohol-drinking behavior in humans, previous studies have investigated the role of additional NMDA-receptor subtypes and observed an association of genetic variations of *NR1* with alcohol dependence and *NR2B* with Cloninger type 2 alcoholism.[54] This finding could be replicated in part in a study that observed an association of *NR1* only in patients with a history of withdrawal-induced seizures.[55] Given the hypothesis of glutamatergic hyperexcitability in alcohol withdrawal, the observed discrepancy may be a result of genetic heterogeneity.[56] In this example, selection of withdrawal-related phenotypes may have enriched for a neurobiological mechanism that is particularly sensitive to alterations in glutamatergic function and thus identified an association, which in this sample was not detectable on analysis of the more heterogenous phenotype of alcohol dependence.

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European studies—including our current work—did not find additional evidence of an association between alcohol dependence and *NR2B* in rs1806201.[57,58] However, a recent study from South Korea reports an association of the same genetic variation with alcohol dependence.[59] Allelic distributions of rs1806201 in the HapMap database are 0.22 in Europeans and 0.54 in Asians, suggesting significant ethnic differences, which may contribute to the observed discrepant results.

This phenomenon may also have contributed to conflicting results of a genetic variation of the protein tyrosine kinase *FYN* gene, which was found to be associated with alcohol dependence in a German[60] but not a Japanese[61] sample. Further analysis of this gene is relevant, as *Fyn* knockout mice show increased alcohol sensitivity and lack of tolerance to the effects of ethanol[62] owing to a reduction of fyn-dependent phosphorylation of NR2A.[63]

While in the first step of our analysis, both *NR2A* and *MGLUR5* were shown to be associated with alcohol dependence; our replication analysis did not support an association of *MGLUR5* with alcohol dependence. This may have been a consequence of a limitation of the study: controls in study 2 were selected on the basis of a questionnaire as opposed to controls in study 1, who were selected on the basis of a diagnostic interview, which may have led to the erroneous inclusion of alcohol-dependent participants in the control sample and resulted in decreased power in the second study to detect differences between cases and controls. An absence of association between *MGLUR5* and alcohol dependence stands in contrast to results from animal studies, which clearly identify a role of mGluR5 in alcohol-related behavior. In pharmacological studies using the mGluR5 antagonist 2-methyl-6-phenylethynylpyridine, the relevance of this gene for alcohol self-administration and relapse behavior in rats[17,19] and mice[18] has been established. This discrepancy may suggest that the role of mGluR5 is limited to specific (intermediate) phenotypes, which the current study was unable to identify, or it may indicate an involvement of a gene farther downstream in the mGluR5-signaling cascade, which so far has not been implicated in the behavioral effects observed in animal studies.

Interestingly, we observed only an association with alcohol dependence in genes encoding transmembrane proteins but not in intracellular signaling genes. This is somewhat unexpected, as various animal models have shown pronounced effects on alcohol-drinking behavior mediated by genes encoding intracellular proteins, such as neuronal nitric oxide synthase[27] and cyclic guanosine monophosphate–kinase II.[28] While this may be in part explained by a greater complexity of the diagnosis of alcohol dependence compared with behavioral animal paradigms of alcohol-drinking behavior, it may also indicate a lack of specificity of knockout models, whereby deletion of a single gene may lead to inactivation of an entire signaling cascade.

The limited analogy of knockout models and analysis of human genetic variations clearly constitutes one of the limitations of the translational claim of studies such as the one presented. Other limitations of our study result from the parsimonious selection of genetic variations. While designed to cover the most frequent haplotypes selected for functionally relevant genetic variations, this analysis does not consider potentially functional SNPs in intronic enhancer sequences nor does it take into account a variable number of tandem repeats in the genes analyzed.[64] Based on the robust results generated from our large data set, further genotype-specific molecular characterizations of *NR2A* may result in its application for pharmacogenetic studies of response to the anticraving drug acamprosate as well as providing a potential target for pharmacological interventions in human alcohol dependence.

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Correction

Error in Byline. In the Original Article by Eaton et al titled "Population-Based Study of First Onset and Chronicity in Major Depressive Disorder," published in the May issue of the *Archives* (2008; 65[5]:513-520), there was an error in the byline. The name that appeared as Ben Hochang Lee should have read Hochang Benjamin Lee.

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