Eur Arch Psychiatry Clin Neurosci (2005) 255 : 129–135 DOI 10.1007/s00406-004-0545-7

ORIGINAL PAPER

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Polymorphisms in the NMDA subunit 2B are not associated with alcohol dependence and alcohol withdrawal-induced seizures and delirium tremens

Received: 19 May 2004 / Accepted: 18 August 2004 / Published online: 19 November 2004

Abstract *Objective* Ethanol-inhibited glutamatergic neurotransmission has been shown to mediate pathophysiological mechanisms in the development of alcoholism, including withdrawal symptoms. NMDA-receptor 2B (NR2B) is a subunit that confers a high sensitivity to ethanol-induced inhibition. Previously we had reported a lack of association between the single nucleotide polymorphism (SNP) rs1806201 in the NR2B gene *(GRIN2B)* and alcoholism. Shortly thereafter, an association between the polymorphism and early-onset alcoholism has been reported. One aim of the present study was to test whether the association between the *GRIN2B* polymorphism rs1806201 and early-onset alcoholism can be replicated in a larger sample. Moreover, we hypothesized that another genetic variation within *GRIN2B* (rs1806191) may have an effect in the etiology of alcoholism or withdrawal-related traits. *Methods* We extended our original study sample to a size of 377 patients and 464 healthy volunteers and performed a replication study, including the second *GRIN2B* SNP. Associations between allele, genotype and haplotype frequencies of the two polymorphisms and alcoholism as well as with patients' phenotypes were investigated. *Results* No associations were found between any of the two polymorphisms, tested individually or as haplotypes, and alcoholism, respectively withdrawal-related traits. *Conclusion* Neither the analyzed SNPs nor any of their haplotypes likely modify susceptibility to alcohol dependence or withdrawal-related phenotypes.

Key words NMDA receptor · NR2B · GRIN2B · alcohol dependence · alcohol withdrawal

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The study was supported by a MAIFOR grant of the University of Mainz (AT, ND, AS) and by the Bundesministerium für Bildung und Forschung, BMBF, Grant No. 01EB0140–42 (MS)

Introduction

Neuropharmacological studies have underlined the importance of glutamatergic neurotransmission for the development of alcoholism. Ethanol-inhibited glutamatergic neurotransmission has been shown to mediate pathophysiological mechanisms in the development of alcoholism and its physical consequences, including tolerance, withdrawal symptoms, craving and ethanol-related neurotoxicity (for reviews, see Fadda and Rosetti 1998; Tsai and Coyle 1998).

Patch-clamp studies in Xenopus oocytes reported direct ethanol-protein interaction for the *N*-methyl-D-aspartate (NMDA) receptor (Wright et al. 1996). On this receptor, which mediates the post-synaptic excitatory effects of glutamate or glycine, ethanol interacts with an allosteric site and reduces agonist efficacy by modulat-

ing the kinetics of channel gating (Wright et al. 1996). Chronic ethanol exposure upregulates NMDA receptor function (Gulya et al. 1991), increases receptor-mediated function (Iorio et al. 1992), and induces changes of NMDA receptor subunit composition (Gulya et al. 1991). In the status of acute ethanol withdrawal up-regulated NMDA-receptors contribute to ethanol tolerance and are associated with an increased excitability that is thought to mediate clinical symptoms such as seizures, delirium tremens and neuronal cell death leading to brain atrophy (Tsai et al. 1995). Moreover, both high-affinity NMDA-antagonists such as ketamine and low-affinity antagonists such as dextrometorphan show ethanol-like effects in detoxified alcoholics (Krystal et al. 1998, 2003; Schuetz and Soyka 2000; Soyka et al. 2000). The weak NMDA receptor antagonist acamprosat (Rammes et al. 2001) has been used for relapse prevention in alcohol dependence. This anti-craving compound showed effects both in rodents and humans (Johnson and Ait-Daoud 2000; Sass et al. 1996; Spanagel and Zieglgänsberger 1997) and is supposed to reduce the aversive effects of alcohol withdrawal by inhibiting NMDA receptor-mediated hyperexcitability (Spanagel et al. 1996).

Although allosteric inhibition of NMDA-receptor activity is a general pharmacological effect of ethanol (Peoples and Stewart 2000), its degree of ethanol-induced inhibition is related to the subunit composition of the NMDA receptor (Masood et al. 1994). NMDA-receptor 2B (NR2B) is a subunit that confers a high sensitivity to ethanol-induced inhibition (Masood et al. 1994). Ethanol reduces NMDA-evoked release of various neurotransmitters, particularly dopamine, noradrenaline, serotonine and GABA in a variety of brain regions, largely by blockade of NMDA receptors with a high proportion of NR2B subunit (Fink and Gothert 1996). Mesencephalic neurons which have been implicated in mediating the reinforcing effects of drugs of abuse (Spanagel and Weiss 1999), including alcohol, bear predominantly NMDA receptors with the ethanol-sensitive NR2B subunit (Allgaier et al. 1999). This subunit was reported to be selectively up-regulated – particularly in the nucleus accumbens and hippocampus – in CRH1-deficient mice, which showed an enhanced and delayed stress-induced alcohol intake (Sillaber 2002). Taken together, these findings suggest a direct involvement of the NR2B subunit in ethanol-altered NMDA-receptor signaling as well as in alcohol dependence.

Alcohol dependence is a disorder with strong genetic influences and heritability estimates range between 50–60 % (Kendler et al. 1994; Johnson and Ait-Daoud 2000; Prescott and Kendler 1999). Due to polygenic inheritance, genetic variability of single candidate genes may account for a part of the variance determining susceptibility and pathophysiology of alcoholism.

Association studies between polymorphisms within the NR2B receptor gene (chromosome 12p13.1) and alcoholism are scarce. We are aware of two investigations in independent Caucasian samples of the silent single nucleotide polymorphism (SNP) rs1806201 (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rs&rs=1806201; C2873T; Thr888Thr) in the gene region (exon 13) encoding the carboxyl-terminal intracellular domain of the NR2B subunit (Nishiguchi et al. 2000), which revealed conflicting results. Our own finding of no association between this polymorphism and alcohol dependence in a sample of 204 alcoholic patients and 258 controls (Schumann et al. 2002) was contradicted by Wernicke et al. (Wernicke et al. 2003), who found an association between a reduced T allele prevalence and an early onset of alcohol dependence.

The first aim of the present study was to test whether the aforementioned association between the non-functional NR2B polymorphism rs1806201 and early onset of alcohol dependence could be replicated in a larger sample of patients and healthy volunteers and to analyze withdrawal related traits. Towards this end, we extended our original study to a size of 377 patients and 464 healthy volunteers (both of Middle European descent). Second, we extended the analysis by investigating the possible association between a further silent A/G-SNP (rs1806191; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rs&rs=1806191; A3743G; His1178His) within the same gene region (exon 13), 870 bp downstream of rs1806201. This SNP was chosen because of its highly informative character due to a heterozygosity of about 50 %.

Material and methods

Subjects and psychometry

The case control sample consisted of 377 alcohol-dependent patients and 464 unrelated healthy volunteers, who were recruited in two university centers. All patients and healthy volunteers were of Caucasian descent.

One-hundred and ninety-six alcohol-dependent patients (149 males, 47 females) with a mean age of 43.7 (±8.7) years were recruited at the Department for Psychiatry of the University of Mainz, following their admission to the hospital for alcohol withdrawal. The diagnosis of alcohol dependence was determined by an experienced psychiatrist (IA, CK or AS) according to DSM-IV criteria (American Psychiatric Association 1994) and was confirmed by the standardized interview M-CIDI (Wittchen et al. 1998). Exclusion criteria were psychotic disorders such as schizophrenia and other substance abuses or dependences, except for nicotine abuse or dependence. Symptoms related to alcohol dependence were examined by means of the Michigan Alcoholism Screening Test (MAST), a 25-item structured, self-rating instrument (Selzer 1971). A further 181 alcohol-dependent patients (142 males, 39 females) with a mean age of 41.1 (±9.3) were recruited after admission for alcohol withdrawal to the addiction treatment ward of the Department of Psychiatry, Ludwig-Maximilians University of Munich. They both met ICD-10 (World Health Organization 1992) and DSM-IV criteria of alcohol dependence determined by SCID I and the SSAGA interview (Buchholz et al. 1994; Hesselbrock et al. 1999) and a comprehensive psychiatric interview was performed by an experienced psychiatrist (GK, UWP or MS). Patients with concomitant Axis I disorders were excluded. Details of patients in the Munich sample have been given elsewhere (Preuss et al. 2000, 2001; Soyka et al. 2002). All patients from both centers were assessed after alcohol withdrawal approximately two weeks after admission.

The majority of the controls were randomly selected from the general population of Munich. After the first contact by mail, volun-

teers responded by phone and were further screened before inclusion in the study in order to exclude neuropsychiatric disorders in the volunteers and their first-degree relatives. Subsequently, they were assessed by means of detailed medical and psychiatric history forms for themselves and their first-degree relatives. SCID I and SCID II questionnaires (Wittchen et al. 1997), structured clinical interviews based on DSM-IV criteria, were performed. The Family History Assessment Module (Rice et al. 1995) was used to examine psychiatric history of first-degree relatives. Volunteers with a positive history of psychiatric disorder in themselves or in a first-degree relative were not included in the study. This control group consisted of 386 healthy Middle Europeans (172 males, 214 females) with a mean age of 45.5 (±14.9) years. Another 78 healthy Caucasians of Middle European origin (54 males, 24 females) with a mean age of 41.6 (±11.5) years were recruited in the city of Mainz through notice board advertisement.

Ethical standards

After a complete and extensive description of the study all patients and controls gave their written informed consent to participate in the study. The local ethical committees of the Landesärztekammer Rheinland-Pfalz and the Ludwig-Maximilians University, respectively, approved the study.

Genotyping

Genomic DNA was extracted from blood samples using standard extraction methods. Genotyping of two synonymous single nucleotide polymorphisms (SNPs), rs1806201 (http://www.ensembl.org/Homo_sapiens/snpview?snp=1806201; *C2873T; Thr888Thr*) and rs1806191 (http://www.ensembl.org/Homo_sapiens/snpview?snp=1806191; *A3743G; His1178His*), in exon 13 of *GRIN2B* were performed by Pyrosequencing™ (Alderborn et al. 2000). The following primer pairs were used for PCR amplification of the SNP containing fragment and subsequent real time sequencing: 1) rs1806201: 5'-biotin-TTCACACCAGACAGGTTAGC-3' (reverse) and 5'-AGCGCCAGTCTGTAATGA-3' (forward), sequencing primer: 5'-AATGAACTCCCCCAC-3' (forward assay); and 2) rs1806191: 5'-biotin-GACCAGTTCCGAACAAAGGA-3' (forward) and 5'-TTCTCCCAAGGTGCAGGTA-3' (reverse), sequencing primer: 5'-CGTCCCGTGCTTGAT-3' (reverse assay). The biotinylated strand of the PCR product was immobilized onto streptavidin-coated paramagnetic beads (Dynabead sM280; Dynal, Oslo, Norway) and a single stranded immobilized DNA fragment was obtained after NaOH denaturation. Automated real-time sequencing was conducted at room temperature in a volume of 25 µl on a 96-well PSQ™ 96 instrument using enzymes and substrates from the PSQ 96 SNP reagent kit (Pyrosequencing AB, Uppsala, Sweden).

Statistics

Statistical analyses were performed using SPSS software (Statistical Package for Social Sciences, Version 10.0, SPSS Inc, Chicago, 1999). One-way ANOVA or χ^2-statistics were performed, as appropriate for the measurement, to test for differences between genotype groups and between diagnostic subgroups and controls. Hardy-Weinberg equilibrium was tested in each subgroup by comparing the observed and expected genotype frequencies by two-tailed χ^2-statistics. A two-tailed type I error rate of 5 % was chosen for the analysis. Power estimates (*power* defined as the chance that true differences will actually be detected) were made using the power calculation software of the UCLA Department of Statistics (http://ebook.stat.ucla.edu/calculators/powercalc). Haplotype frequencies were estimated by maximum likelihood estimation based on expectation maximization (EM) algorithm, using the COCAPHASE software version 2.40 from the UNPHASED software package (Dudbridge 2003). Haplotypes with a frequency lower than 1 % were dropped. With the same program, differences in haplotype distribution between patients and controls were assessed with 10,000 simulations (Sham and Curtis 2000) as well as the calculation of the normalized linkage disequilibrium coefficient *D'* and the squared correlation coefficient r^2.

Results

A total of 377 inpatients (291 males and 86 females, mean age ± SD = 42.4 ± 9.1 years) meeting DSM-IV criteria for alcohol dependence were recruited. As a comparison group, 464 healthy volunteers (226 males and 238 females, mean age ± SD = 44.9 ± 14.5 years) were included in the study. Sample characteristics of alcohol-dependent patients and controls are presented in Table 1. The characteristics of alcohol-dependent patients divided by genotype are presented in Table 2. No differences were detected between genotype groups of each polymorphism.

Table 1 Sample characteristics

	Alcohol-dependent patients	Controls
Sex (males/females)	291/86	226/238
Age (years)	42.4±9.1	44.9±14.5
Age of onset (years)	30.1±9.6	
Duration of dependence (years)	11.5±8.5	
Family history positive (n°; %)	120 (44.0)[1]	
Alcohol withdrawal-induced epileptic seizures (n°; %)	57 (15.1)	
Alcohol withdrawal-induced delirium tremens (n°; %)	64 (16.9)	

[1] Family history data were present in 273 alcohol-dependent patients; *family history "positive"* indicates that at least one first degree relative is affected by alcohol dependence

Table 2 Alcohol-dependent patients characteristics and NR2B polymorphisms genotype

	SNP rs1806191			SNP rs1806201		
	AA	AG	GG	CC	CT	TT
Sex (m/f), n = 377	78/22	143/43	70/21	153/48	120/32	18/6
Age (years)	41.7±8.5	42.7±9.2	42.3±9.6	42.3±8.8	42.9±9.4	40.3±9.9
Age of onset (years)	29.2±9.3	30.4±9.4	30.5±10.2	30.3±8.9	30.0±10.6	29.5±9.0
Duration of dependence (years)	11.9±9.0	11.4±8.3	11.2±8.5	10.9±8.2	12.4±8.5	11.2±10.4

No differences were detected between genotype groups of each polymorphism (one-way ANOVA, $p < 0.05$)

SNP rs1806201

Genotype distribution and allele frequencies in patients and controls are shown in Table 3. The genotype distribution was in Hardy-Weinberg equilibrium among both healthy volunteers ($\chi^2 = 0.1$, df = 1, p = 0.76) and patients ($\chi^2 = 0.45$, df = 1, p = 0.51).

Comparison of genotype and allele distribution between healthy volunteers and alcohol-dependent patients detected no significant differences ($\chi^2 = 0.30$, df = 2, p = 0.86; $\chi^2 = 0.21$, df = 1, p = 0.65). Analysis by gender showed no differences between alcohol-dependent patients and the controls in genotype distribution (males: $\chi^2 = 1.04$, df = 2, p = 0.59; $\chi^2 = 0.15$, df = 1, p = 0.70; females: $\chi^2 = 1.04$, df = 2, p = 0.60; $\chi^2 = 0.36$, df = 1, p = 0.55) as well as between alcohol-dependent males and females ($\chi^2 = 0.46$, df = 2, p = 0.79; $\chi^2 = 0.10$, df = 1, p = 0.75). Subsequent analyses investigated differences between selected alcohol phenotypes as shown in Table 3 and healthy volunteers. Both groups defined by an early (≤ 25 years) or a late (≤ 26 years) age of onset of alcohol dependence showed no significant differences compared to healthy controls (χ^2-values not reported, for p-values see Table 3) and compared to each other.

Moreover, we analyzed whether the SNP rs1806201 could be a risk factor for alcohol withdrawal-induced seizures and delirium tremens. The four subgroups of alcohol-dependent patients were thus defined by a positive or negative history of alcohol withdrawal-induced seizures or delirium tremens. Comparison of genotype distribution and allele frequencies between each subgroup and healthy volunteers showed no significant differences between groups (χ^2-values not reported, for p-values see Table 3). Comparison of genotype distribution and allele frequencies between corresponding subgroups revealed again no significant differences (χ^2-values not reported, for p-values see Table 3).

Comparison of genotype distribution and allele frequencies between subgroups with or without a positive family history revealed no significant differences. Only the comparison of patient subgroups showed a trend towards an over-representation of the CC genotype in patients with affected first degree relatives ($\chi^2 = 5.42$, df = 2; p = 0.067) and a significant analogous difference of allele frequencies ($\chi^2 = 5.25$; df = 1; p = 0.022). Given the numerous comparisons of allele frequencies (16 within the analysis of SNP rs1806201) correction for multiple testing was performed. After this Bonferroni adjustment the result was no more statistically significant (see Table 3).

SNP rs1806191

Genotype distribution and allele frequencies in patients and controls are shown in Table 4. The genotype distribution was in Hardy-Weinberg equilibrium among both healthy volunteers ($\chi^2 = 0.08$, df = 1, p = 0.78) and patients ($\chi^2 = 0.06$, df = 1, p = 0.81).

Genotype distribution and allele frequencies did not differ between alcohol-dependent patients and healthy controls ($\chi^2 = 0.21$, df = 2, p = 0.90; $\chi^2 = 0.07$, df = 1, p = 0.79, respectively). All subsequent comparisons were performed identically to the analysis of SNP rs1806201

Table 3 SNP rs1806201 genotype distribution and allele frequencies in controls and patient subgroups

	Genotype distribution					Allele frequencies			
Sample	CC n (%)	CT n (%)	TT n (%)	P[1] (df = 2)	P[2] (df = 2)	C n (%)	T n (%)	P[3] (df = 1)	P[4] (df = 1)
Controls – total group	256 (55.2)	179 (38.6)	29 (6.3)			691 (74.5)	237 (25.5)		
Patients									
– total group	201 (53.3)	152 (40.3)	24 (6.4)	0.86		554 (73.5)	200 (26.5)	0.65	
– age of onset 25 years	49 (51.6)	39 (41.1)	7 (7.4)	0.79		137 (72.1)	53 (27.9)	0.50	
– age of onset ≥ 26 years	92 (55.4)	60 (36.1)	14 (8.4)	0.59	0.73	244 (73.5)	88 (26.5)	0.73	0.73
– seizures positive	32 (56.1)	24 (42.1)	1 (1.8)	0.38		88 (77.2)	26 (22.8)	0.53	
– seizures negative	150 (54.2)	105 (37.9)	22 (7.9)	0.68	0.24	405 (73.1)	149 (26.9)	0.56	0.37
– delirium positive	39 (60.9)	22 (34.4)	3 (4.7)	0.66		100 (78.1)	28 (21.9)	0.37	
– delirium negative	132 (55.2)	92 (38.5)	15 (6.3)	1.0	0.69	356 (74.5)	122 (25.5)	0.99	0.40
– family history positive	73 (60.8)	42 (35.0)	5 (4.2)	0.46		188 (78.3)	52 (21.7)	0.22	
– family history negative	76 (49.7)	61 (39.9)	16 (10.4)	0.18	0.07	213 (69.6)	93 (30.4)	0.10	0.35[5]
By gender									
Males									
Controls	119 (52.7)	88 (38.9)	19 (8.4)			326 (72.1)	126 (27.9)		
Patients	153 (52.6)	120 (41.2)	18 (6.2)	0.59		426 (73.2)	156 (26.8)	0.70	
Females									
Controls	137 (57.6)	91 (38.2)	10 (4.2)			365 (76.7)	111 (23.3)		
Patients	48 (55.8)	32 (37.2)	6 (7.0)	0.60	0.79	128 (74.4)	44 (25.6)	0.55	0.75

[1] χ^2-test to compare genotype distributions between patient groups and patient subgroups with corresponding control group, df = 2; [2] χ^2-test to compare genotype distributions between corresponding patient subgroups, df = 2; [3] χ^2-test to compare allele frequencies between patient groups and patient subgroups with corresponding control group, df = 1; [4] χ^2-test to compare allele frequencies between corresponding patient subgroups, df = 1; [5] Level of significance after Bonferroni-correction for multiple testing (factor 16)

Table 4 SNP rs1806191 genotype distribution and allele frequencies in controls and patient subgroups

Sample	Genotype distribution					Allele frequencies			
	AA n (%)	AG n (%)	GG n (%)	P[1]	P[2]	A n (%)	G n (%)	P[3]	P[4]
Controls – total group	117 (25.2)	235 (50.6)	112 (24.1)			469 (50.5)	459 (49.5)		
Patients									
– total group	100 (26.5)	186 (49.3)	91 (24.1)	0.90		386 (51.2)	368 (48.8)	0.79	
– age of onset 25 years	28 (29.5)	45 (47.4)	22 (23.2)	0.69		101 (53.2)	89 (46.8)	0.51	
– age of onset ≥26 years	40 (24.1)	81 (48.8)	45 (27.1)	0.75	0.59	161 (48.5)	171 (51.5)	0.52	0.31
– seizures positive	18 (31.6)	24 (42.1)	15 (26.3)	0.44		60 (52.6)	54 (47.4)	0.67	
– seizures negative	72 (26.0)	137 (49.5)	68 (24.5)	0.95	0.57	281 (50.7)	273 (49.3)	0.95	0.71
– delirium positive	18 (28.1)	32 (50.0)	14 (21.9)	0.86		68 (53.1)	60 (46.9)	0.58	
– delirium negative	63 (26.4)	115 (48.1)	61 (25.5)	0.82	0.83	241 (50.4)	237 (49.6)	0.97	0.59
– family history positive	33 (27.5)	66 (55.0)	21 (17.5)	0.30		132 (55.0)	108 (45.0)	0.22	
– family history negative	38 (24.8)	66 (43.1)	49 (32.0)	0.13	0.35[5]	142 (46.4)	164 (53.6)	0.21	0.74[5]
By gender									
Males									
Controls	56 (24.8)	111 (49.1)	59 (26.1)			223 (49.3)	229 (50.7)		
Patients	78 (26.8)	143 (49.1)	70 (24.1)	0.81		299 (51.4)	283 (48.6)	0.52	
Females									
Controls	61 (25.6)	124 (52.1)	53 (22.3)			246 (51.7)	230 (48.3)		
Patients	22 (25.6)	43 (50.0)	21 (24.4)	0.91	0.98	87 (50.6)	85 (49.4)	0.80	0.85

[1] χ^2-test to compare genotype distributions between patient groups and patient subgroups with corresponding control group, df = 2; [2] χ^2-test to compare genotype distributions between corresponding patient subgroups, df = 2; [3] χ^2-test to compare allele frequencies between patient groups and patient subgroups with corresponding control group, df = 1; [4] χ^2-test to compare allele frequencies between corresponding patient subgroups, df = 1; [5] Level of significance after Bonferroni-correction for multiple testing (factor 16)

(see above). After Bonferroni correction no significant differences between groups were detected (see Table 4).

Haplotype analysis

Haplotype frequencies for the two *GRIN2B* polymorphisms are shown in Table 5. Both alcohol-dependent and control samples showed similar haplotype frequencies.

Among the four possible haplotypes, three were estimated to occur with frequencies greater than 1 % in both samples. Comparing whole groups, the likelihood ratio test indicates that the two samples do not differ in their haplotype frequencies ($\chi^2 = 0.43$, df = 2, p = 0.81). Besides, strong linkage disequilibrium was found for the haplotypes both in cases and controls (r^2 for cases = 0.3623; r^2 for controls = 0.3402; *D'* for cases and controls = 1), as one can expect regarding the small distance of 870 bp between the two analyzed SNPs. The comparison of haplotype frequencies between controls and patients' subgroups also did not detect any significant differences (see Table 5).

Table 5 Haplotype distribution in controls and patient subgroups

Sample	A-C n (%)	G-C n (%)	G-T n (%)	P[1]
Controls – total group	462 (50.2)	226 (24.6)	232 (25.2)	
Patients				
– total group	377 (50.7)	173 (23.3)	194 (26.1)	0.81
– age of onset 25 years	95 (51.6)	39 (21.2)	50 (27.2)	0.60
– age of onset ≥26 years	160 (48.5)	84 (25.5)	86 (26.1)	0.86
– seizures positive	60 (52.6)	28 (24.6)	26 (22.8)	0.84
– seizures negative	274 (50.2)	128 (23.4)	144 (26.4)	0.84
– delirium positive	66 (52.4)	33 (26.2)	27 (21.4)	0.64
– delirium negative	236 (50.0)	118 (25.0)	118 (25.0)	0.98
– family history positive	128 (54.2)	58 (0.26)	50 (0.22)	0.39
– family history negative	141 (0.46)	72 (0.24)	91 (0.30)	0.27
By gender				
Males				
Controls	218 (48.9)	106 (23.8)	122 (27.4)	
Patients	293 (50.9)	130 (22.6)	153 (26.6)	0.81
Females				
Controls	244 (51.5)	120 (25.3)	110 (23.2)	
Patients	84 (50.0)	43 (25.6)	41 (24.4)	0.94

[1] Likelihood ratio test to compare patient groups and patient subgroups with corresponding control group (df = 2, best P-value after 10,000 permutations)

Discussion

In our study, the associations between alcohol-related traits and two polymorphisms within the gene coding for the NMDA subunit 2B as well as their haplotypes were investigated.

In a previous investigation of the SNP *rs1806201* in a subsample of our alcohol-dependent patients and healthy volunteers we found no differences between groups (Schumann et al. 2002). This finding was recently contradicted by Wernicke et al. (Wernicke et al. 2003), who found a reduced T-allele frequency a) in alcohol-dependent patients with an age of onset ≤ 25 years and b) in Cloninger type 2 alcoholics, characterized by an early age of onset ≤ 25 years, dissocial personality disorder and male gender. In the present study we found no

overall differences between patients and controls (power > 99 %, assumed RR = 2.0), which is in line with the above mentioned previous studies concerning the influence of this SNP on alcoholism.

Moreover, after stratification by age of onset (≤ 25 years, ≥ 26 years) we detected no significant differences in genotype distribution and allele frequencies compared to healthy controls or between age of onset groups. The power of our subsamples (age of onset ≤ 25 years, n = 95) was > 90 % (C-allele = risk factor) to detect a genetic factor conferring even a low relative risk (RR) of 2.0, but decreased to 21 % assuming a relative risk of 1.243 (RR = 1.243 calculated on the data basis from the Wernicke paper). This represents a limitation of our study; we would have needed a sample size of n = 1369 patients and n = 1369 controls in order to achieve a power of 80 % to detect a genetic risk factor conferring a relative risk of 1.243. Nevertheless, our data argue against the hypothesis that the SNP rs1806201 plays a relevant role in early onset alcoholism.

The association analyses of subgroups of selected patient phenotypes, representing groups with a positive or negative history of alcohol withdrawal-induced seizures showed in each case no significant differences compared to healthy volunteers. Both samples of patients with a *positive* history of alcohol withdrawal-induced seizures or delirium tremens had a power between ~60 % (C-allele assumed as the risk factor, RR = 2.0) and ~75 % (T-allele assumed as the risk factor, RR = 2.0). Again, this moderate power represents a limitation of our study: in our sample the relative risk conferred by the SNP1806201 C-allele would have needed to exceed 2.6 (T-allele: RR > 2.1) to be detected with a power of 80 % at a single-sided alpha-level of $\alpha = 0.05$. On the other hand, a sample size between n = 125 and n = 187 patients (delirium positive, C-allele as risk factor: n = 187; delirium positive, T-allele as risk factor: n = 128; seizures positive: C-allele as risk factor: n = 180 patients, T-allele as risk factor: n = 125) would have been necessary to detect a genetic risk factor conferring a relative risk of 2.0 with a power = 80 %. However, the samples of patients with a *negative* history of alcohol withdrawal-induced seizures or delirium tremens had a power of > 96 % (RR = 2.0), but no differences were detected between groups. Thus, this SNP might not be involved in the genetic susceptibility to alcohol withdrawal-induced seizures and delirium tremens.

The novel silent A/G mutation *rs1806191* within the NR2B was, as far as we know for the first time, investigated because of its potential highly informative character due to a heterozygosity of about 50 %. No overall differences were detected in genotype or allele frequencies between patients and controls in our large case control sample with a power > 99 % to detect a genetic factor conferring even a low relative risk (RR) of 2.0. Thus, our finding does not support the hypothesis that the SNP rs1806191 plays a significant role in the genetic susceptibility of alcohol dependence. Division of the patient sample by age of onset (age of onset ≤ 25 years: power > 90 %, assumed RR 2.0; age of onset ≥ 26 years: power > 98 %, assumed RR 2.0) and analysis of genotype and allele frequencies, again, revealed no differences between both subgroups and controls. This finding does not support the idea that the SNP rs1806191 influences the age of onset of alcoholism. The association analyses of subgroups of selected patient phenotypes, representing groups with a positive or negative history of alcohol withdrawal induced seizures showed in each case no significant differences compared to healthy volunteers. The samples of patients with a *positive* history of alcohol withdrawal-induced seizures or delirium tremens had each a power of ~ 80 % to detect a genetic factor conferring a relative risk of 2.0; the samples of patients with a *negative* history of alcohol withdrawal-induced seizures or delirium tremens had even a power of > 99 % (RR = 2.0). Since no differences were detected between groups, this SNP most likely plays no major role in the genetic susceptibility to alcohol withdrawal-induced seizures and delirium tremens.

The investigation of the influence of both SNPs on the presence or absence of positive family history of alcohol dependence revealed no significant differences between patient subgroups and controls. It is noteworthy that the comparison of patient subgroups revealed weakly significant differences between genotype distribution (SNP rs1806191) and allele frequencies (both SNPs), which, however, turned insignificant after Bonferroni correction. Thus, the exclusion of rs1806201 and rs1806191 as potential risk factors for the development of family history positive alcoholism seems less decisive compared to the other investigated phenotypes.

Moreover, no overall differences were detected in haplotype distribution between patients and controls in our large case control sample.

Thus, our findings argue against the hypothesis that any of the two investigated polymorphisms or their haplotypes play a major role in the genetic susceptibility to alcohol dependence or to withdrawal-related traits. This conclusion is in line with results from several linkage studies for the genetics of alcoholism, which did not find positive linkage results at 12p13.1 (for review see: Tyndale 2003). Moreover, the results reinforce our previous finding of a lack of association between the *GRIN2B* polymorphism rs1806201 with alcohol-related traits. However, the gene itself should not be totally dismissed as a candidate gene as our study has not addressed all potentially relevant endophenotypes or subtypes of alcoholism and given the involvement of the gene product in alcohol relevant mechanisms, the rather large size (> 400 kB, 13 exons) of the gene and the abundance of its genetic variability (> 800 SNPs).

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