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ORIGINAL PAPER

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Alcoholism-related phenotypes and genetic variants of the CB1 receptor

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Abstract *Objective* Neurotransmitter release of GABAergic and glutamatergic neurons may be significantly influenced by cannabinoid CB1 receptors located at presynaptic nerve terminals. GABA and glutamate have been reported to be involved in the pathogenesis of severe alcohol withdrawal-induced seizures and delirium tremens. The aim of this study is to test the potential influence of a bi-allelic cannabinoid receptor gene (CNR1) polymorphism (G1359A) on severe alcohol-withdrawal syndromes. *Methods* Based upon a sample size estimation, 196 subjects meeting DSM IV and ICD10 criteria for alcohol dependence and 210 non-alcoholic controls were recruited for study. CB1 polymorphisms were determined using polymerase chain reaction (PCR). History of alcohol withdrawal-induced delirium tremens, seizures and other alcohol withdrawal-related phenotypes were obtained using the SSAGA (Semi-Structured Assessment of Genetics in Alcoholism). Data were corroborated with information from the inpatients' clinical files. *Results* Allele frequencies of the CNR1 G1359A polymorphism were within the range reported by previous studies. After correcting for multiple testing, no association of the A- or G-allele of CNR1 polymorphism with a history of alcohol withdrawal-induced seizures was detected. In addition, no significant relationships with other alcoholism-related phenotypes were found. *Conclusion* This study failed to confirm an earlier report of a potential role of a CNR1 polymorphism in the pathogenesis of delirium tremens.

Key words alcohol dependence · alcohol withdrawal · alcoholism-related phenotypes · cannabinoid 1 receptor · gene polymorphism

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Introduction

Accumulated evidence suggests that endogenous cannabinoids function as diffusible and short-lived intercellular messengers that modulate synaptic transmission. Recent studies reported strong experimental evidence that endogenous cannabinoids provide a retrograde mediation of signals from depolarized postsynaptic neurons to presynaptic terminals and suppress subsequent neurotransmitter release, driving the synapse into an altered state (Elphick and Egertova 2001; Giuffrida et al. 2001).

The main molecular targets for those endogenous cannabinoids, as well as the active component delta(9)-tetrahydrocannabinol of marijuana and hashish, are cannabinoid receptors. They are members of a major family of G protein-coupled seven-transmembrane-domain receptors. Until now, two subtypes of cannabinoid receptors have been reported (Mechoulam 2000): type 1 (CB1, Gerard et al. 1991; Matsuda et al. 1990) and type 2 (CB2, Munro et al. 1993) receptor. While the CB2 receptor is predominantly found in the immune system where it is expressed in B and T cells (Gurwitz and Kloog 1998), the CB1 is mainly expressed in brain tissues that influence a number of key functions including mood, motor coordination, autonomic function, memory, sensation and cognition. Expression of CB1 is abundant in hippocampus, cerebral cortex, some olfactory regions, caudate, putamen, nucleus accumbens and the cerebellum (Onaivi et al. 2002). CB1 receptors may play a key function in mediating inhibitory effects on GABAergic and glutamatergic neurons (Schlicker and Kathmann 2001). These neurotransmitter systems have been hypothesized to be involved in the pathophysiology of epileptic seizures and delirium due to an up-regulation of glutamate and dopamine and a down-regulation of GABA during alcohol withdrawal (Schmidt and Sander 2000; Soyka 1997).

Particularly in hippocampal neurons, depolarization of postsynaptic neurons and subsequent elevation of

Ca(2+) may lead to transient suppression of GABA-transmitter release (Maejima et al. 2001). Hippocampal kindling has been hypothesized to play an important role in both the pathogenesis of alcohol withdrawal-induced seizures and delirium tremens (Goodwin et al. 2000).

Furthermore, many drugs of abuse, including alcohol and cannabis, increase dopamine release in the nucleus accumbens, affecting the mesolimbic dopaminergic reward system. The facilitatory effect of these substances of abuse on dopamine release could theoretically be explained by activation of cannabinoid CB1 receptors located at presynaptic terminals. There is further support for the involvement of CB1 in alcohol withdrawal from animal studies, where the absence of alcohol withdrawal symptoms was demonstrated in a CNR1 Knockout mouse model (Racz et al. 2003).

Polymorphisms of the CB1 gene (CNR1), located at 6q 14–15 (Hoehe et al. 1991), and their relationship to psychiatric phenotypes have been reported. A triplet repeat marker for CNR1 consisting of nine alleles containing (AAT) 12–20 repeat sequences was identified by Dawson (1995). Comings et al. (1997) hypothesized that this microsatellite might be associated with increased susceptibility to alcohol or drug dependence. They reported an association of this genetic variant of CNR1 with a number of different types of drug dependence (cocaine, amphetamine, cannabis) and intravenous illicit drug use, but found no associations with variables related to alcohol use disorders in non-Hispanic Caucasians. In addition, they also reported a significant relationship between this triplet repeat marker and decreased P300 event-related potential amplitudes in subjects with alcohol and substance use disorders (Johnson et al. 1997). However, a subsequent study (Li et al. 2000) of a sample of Chinese heroin-dependent subjects did not find this genetic variant of the CB1 receptor to be related to heroin abuse susceptibility. Also, in contrast to previous research, a recent study (Heller et al. 2001) reported no relationship between either the microsatellite polymorphism (AAT)n or a silent intragenic biallelic polymorphism (1359G/A) of CNR1 with i. v. opiods drug use among 40 opioid addicts in comparison to controls. This silent mutation of CNR1, resulting in the substitution of G to A at nucleotide position 1359 in codon 435 (Thr), is a common polymorphism in the German population (Hoehe et al. 1993; Gadzicki et al. 1999). Population frequencies of 24–32 % for the rarer allele (A) have been reported. This polymorphism was determined by screening the coding exon of CNR1 using PCR single-stranded confirmation polymorphism (SSCP) analysis (Gadzicki et al. 1999).

Based on the findings of Gadzicki et al. (1999), Schmidt et al. (2002) investigated the potential relationship between this polymorphism and alcohol withdrawal phenotypes among 121 severely affected Caucasian alcoholics and 136 most likely non-alcoholic controls, and reported a significant association of the AA genotype with a history of delirium and a severe alcohol withdrawal syndrome. In their analysis, the allelic frequency of CNR1 1359A was 42.1 % in severely affected alcoholics compared to 31.3 % of the control sample.

The aim of the present study was to assess the potential role of this genetic variant of the CB1 receptor in alcoholics with a history of delirium tremens and epileptic seizures.

Materials and methods

Patients

As described in more detail elsewhere (Preuss et al. 2001, 2002), alcohol-dependent inpatients were recruited from consecutive admission to an addiction ward for the treatment of alcohol dependence. All patients were over 18 years of age and met ICD10 and DSM-IV criteria of alcohol dependence as assessed with structured interviews (SCID: Structured clinical interview according to DSM-IV, German version; Wittchen et al. 1997) and SSAGA (Semi-Structured Interview for assessment of genetics in alcoholism; Bucholz et al. 1995; Hesselbrock et al. 1999). In addition, all subjects underwent a comprehensive psychiatric examination by one of the authors (UWP, GK or MS). Patients with other current Axis I disorders were excluded.

All patients were investigated two weeks after admission, post-alcohol withdrawal and were free of any psychopharmacological treatment including benzodiazepines or other medication to alleviate alcohol withdrawal, neuroleptics or antidepressants. The age of onset of alcohol dependence was defined as the mean of the retrospectively obtained ages of onset of DSM-IV criteria reported in SSAGA. These include higher consumption of alcohol than intended, attempts to stop or control alcohol consumption, significant time spent consuming alcohol or recovering from alcohol intake, regular withdrawal symptoms during important daily obligations like school or work, reduction of important occupational or personal activities because of alcohol intake, continued alcohol consumption despite the occurrence of psychological or physical harm, and occurrence of 50 % higher tolerance to alcohol effects. Average daily alcohol intake based upon the typical daily average alcohol consumption reported for the week immediately prior to admission. Pure alcohol intake was computed in grams/day. Duration of alcohol dependence was computed as the difference between the subject's current age and the age of onset of DSM-IV alcohol dependence.

The frequency of recent DSM-IV alcohol-withdrawal symptoms and DSM-IV alcohol-dependence criteria was computed. Similarly the number of alcohol withdrawal-induced seizures (ICD 10: F10.31 or F10.42) and delirium tremens (ICD 10: F10.4) and their age of onset were identified using the SSAGA. The data were corroborated using information from the patients' files.

Control group subjects were recruited from the general population from a variety of locations (e. g. libraries, road work, large stores) and represented different socioeconomic groups ranging from unskilled workers to university graduates. All control subjects completed a comprehensive medical and psychiatric assessment, including routine laboratory screening, to exclude persons with severe physical or Axis I or Axis II psychiatric disorders such as schizophrenia, depression personality disorders and substance use disorders (including alcohol dependence).

Personality traits were evaluated using the MMPI (Minnesota Multiphasic Personality Inventory, Endler et al. 1989), a personality questionnaire commonly used in clinical practice. All persons reporting a first-degree relative affected with any Axis I disorder or alcohol dependence were excluded.

To avoid data analytic problems due to ethnic stratification, all alcoholics and controls were Caucasians from southern Germany.

Genotyping

Genomic DNA was extracted from leukocytes using standard methods. The PCR was based upon the sequence embl: hsu73304, where the substitution from G to A is located at position 1480. A 257 bp PCR-product was amplified using following primers: Forward: 5'-CTG AGG AGT AAG GAC CTG CG-3' (1316–1335) and Reverse: 5'-TCT TTT CCt GTG CTG CCA G-3' (1572–1554). PCR was carried out in a final volume of 10 µl consisting of 50 ng of genomic DNA, 0.5 µM of each Primer, 200 µM of each dNTP and 0.5 U of Ampli Taq Gold-Polymerase (PE). Amplification was performed for 10 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension 72 °C for 60 s and a final period of extension at 72 °C for 10 min.

After amplification 0.2 µM of each hybridization probe, labelled with LC Red640 for the acceptor (5'-LC Red640-AGG TAA CCA TGT CTG TGT CCA CAG ACA CGT -p (1494–1523)), and fluoresceine for the donor (5'-CAA GAG CAC GGT CAA GAT TG-X (1471–1490)) respectively (FRET principle) was added. The melting curve determination was performed using the Rotorgene (Corbett Research) using the following steps: 95 °C for 15 s, 45 °C for 30 s and gradually raising the temperature to 75 °C with a hold at each temperature for 3 s. The analysis identified two different melting temperatures: at approximately 57 °C for the mismatch (A-allele), and at 62 °C for the complete match (G-allele). A detailed description of the theoretical background and methodology is given by Toyota et al. (2000).

Statistics

All continuous values were tested for normal distribution. The between-group relationships between current age, age of onset of alcohol dependence criteria, duration of alcohol dependence, number of DSM IV criteria reported, number of lifetime alcohol withdrawal symptoms, and average daily alcohol intake were computed using one-way analysis of variance (one-way ANOVA).

The association between the frequencies of seizures and delirium tremens with CB1 genotypes was tested using χ^2 statistics. A two-tailed α-level of $p < 0.05$ was defined to be statistically significant.

Sample size estimation

Using information from Schmidt et al. (2002), which compared 136 healthy men and women and 121 alcohol-dependent subjects regarding the association of severe alcohol withdrawal syndromes and CB1 G1359A genotype, a sample size estimation was made.

They reported an excess of the AA genotype in patients with a history of alcohol-related delirium. A frequency of 11 % of the AA genotype was found in the control group versus a frequency of 24 % for the affected group, odds ratio = 2.45 (95 % CI 1.14–5.245).

Assuming a power $(1-\beta) = 0.9$, $\alpha < 0.05$ and genotype frequencies of Schmidt et al. (2002), the estimated sample size for a case-control study is 196 persons per group (n = 392 for the whole sample).

Ethical standards

Informed consent was obtained from patients and controls following a complete and extensive description of the study. The study was approved by the ethical committee of the Ludwig-Maximilians-University of Munich. All patients provided a written informed consent.

Results

A description of the sample is presented in Table 1. Of the 196 alcohol-dependent subjects recruited, 155 (79 %) were male, n = 45 (23 %) had a history of delirium tremens and n = 31 (16 %) reported a history of alcohol withdrawal-induced epileptic seizures.

Table 1 Sample and alcohol-dependence characteristics

		Alcohol-dependent subjects	Controls
Sex [m/f]	n =	155/41	107/103
Age [years]:		41.11 ± 9.98	42.10 ± 14.15
Age of onset [years]:		29.78 ± 9.07	
Years of alcohol dependence [years]		11.95 ± 8.69	
Daily alcohol intake:		342.4 ± 188.3 [g/d]	
Alcohol withdrawal-induced epileptic seizures [y/n]:		45/151	
Mean age of first epileptic seizure [years]		36.83 ± 8.85	
Alcohol withdrawal-induced delirium tremens [y/n]		31/165	
Mean age of first delirium tremens [years]		34.57 ± 7.71	
Mean number of delirious episodes:		4.17 ± 6.46	
Positive family history of alcoholism [y/n]:		89/107	

m male; *f* female; *g/d* gram per day

The control group of 210 subjects enrolled into the study, of whom 107 (51 %) were male, was similar to the alcoholic subjects in relation to their current age (41.1 ± 9.9 vs. 42.1 ± 14.1 years, t-value: –0.80, p: < 0.42).

Genotype results

Following genotyping, a frequency of 26 % for the A-allele and 74 % for the G-allele, respectively, was observed in the alcoholic sample (Table 2). Homozygosity for the AA-genotype was found in 8 % (n = 15) of alcohol dependent subjects, 37 % had the AG genotype (n = 72) and 55 % (n = 109) were homozygous for the GG genotype. Among controls, the frequency of the A allele was 28 % and 72 % for the G-allele. The frequency of homozygous carriers of the A-allele was 10 % (n = 21); for the G-allele, 36 % were heterozygous (n = 75) and 54 % (n = 114) were homozygous.

Among alcoholic and control subjects, the data followed Hardy-Weinberg equilibrium (χ^2-value = 0.44, df = 2, p = 0.80 and χ^2-value = 2.14, df = 2, p = 0.34) and differed only slightly from data reported by Schmidt et al. (2002, AA: 35 %; AG: 56 %; GG 23 %).

The clinical parameters of age of onset, mean duration of alcohol dependence, number of occurrences of delirium, age of onset of delirium and epileptic seizures, number of DSM-IV alcohol dependence and alcohol withdrawal symptoms and mean alcohol intake showed no significant association with the allelic distributions (see Table 2).

History of alcohol withdrawal-induced seizures and CB1 polymorphism

As demonstrated in Table 3, alcohol-dependent individuals with a history of alcohol withdrawal-induced seizures had a weak relationship with the CB1-G-allele compared to healthy controls and alcoholics without a history of alcohol withdrawal-induced seizures. No association was found between CB1 A or G-allele frequencies and a history of delirium tremens.

Furthermore, to minimize the possibility of finding an association due to the influence of heterozygous allele carriers, an additional analysis was made comparing homozygous AA and GG genotype carriers. However, after applying Bonferroni corrections for multiple testing, the results did not remain statistically significant.

History of alcohol withdrawal-induced delirium tremens and CB1 polymorphism

Alcohol-dependent individuals with a history of alcohol withdrawal-induced delirium tremens showed no excess of the CB1 polymorphism in comparison to the control group and alcoholics without such a history.

Discussion

In the present study, no association was found between the CNR1 G1359A polymorphism and alcoholism-related phenotypes. The phenotypes examined included the number of current withdrawal symptoms, a history of alcohol withdrawal-related delirium and alcohol

Table 2 Alcohol dependence characteristics and CB1 (CNR1)-receptor polymorphism genotypes

	CB1 genotype			ANOVA	
	AA	AG	GG	F-Value	Signif.
Sex [m/f]; n = 196 n =	12/3 (8%)	58/14 (37%)	85/24 (55%)		
Age [years]:	37.40±9.2	40.36±9.4	42.12±10.3	1.81	0.17
Age of onset [years]:	27.77±8.0	29.24±8.9	30.39±9.2	0.66	0.52
Duration of alcohol dependence [years]	11.62±8.3	11.58±8.0	12.25±9.2	0.13	0.88
Daily alcohol intake [g/d]:	281.31±115.9	372.48±210.5	330.38±178.1	1.76	0.18
Number of DSM-IV Withdrawal symptoms	4.85±2.3	5.14±2.4	4.90±2.1	0.24	0.79
No. of delirium tremens episodes (n = 47)	2.00±2.0	7.50±8.8	2.53±4.3	2.67	0.08
No. of DSM-IV Alcohol dependence symptoms	5.23±1.3	5.71±1.2	5.54±1.3	0.79	0.48
Control sample: Sex [m/f] n = 210 n =	12/9 (10%)	44/31 (36%)	51/63 (54%)		

CB1 Cannabinoid receptor 1; *m* male; *f* female

Table 3 The associations of CB1-receptor polymorphisms with seizures and delirium tremens: alcoholics vs. controls

	Controls (n = 210) vs.:					Alcoholics without delirium (n = 165) vs.:				
	χ^2-value	df	OR	95% CI	Signif.	χ^2-value	df	OR	95% CI	Signif.
1. Alcoholics with delirium tremens (n = 31)										
CB1 A-Allele	0.00	1	0.95	0.5–1.9	0.95	0.01	1	1.13	0.5–2.4	0.90
CB1 G-Allele	0.00	1	0.94	0.3–3.3	0.95	0.22	1	0.71	0.2–2.9	0.64
	Controls (n = 210) vs.:					Alcoholics without seizures (n = 151) vs.:				
	χ^2-value	df	OR	95% CI	Signif.	χ^2-value	df	OR	95% CI	Signif.
2. Alcoholics with seizures (n = 45):										
CB1 A-Allele	0.66	1	1.23	0.6–2.3	0.42	0.62	1	1.12	0.8–1.7	0.43
CB1 G-Allele	5.01	1	1.10	0.9–1.6	0.24	4.95	1	0.90	0.8–1.4	0.24
Test for homozygosity effect: epileptic seizures and CB1 AA vs. GG:										
						χ^2-value	df	OR	95% CI	Signif.
Alcoholics with epilepsy (n = 28) vs. controls (n = 136):						5.00	1	1.12	1.1–1.3	0.06
Alcoholics with epilepsy (n = 28) vs. without (n = 104):						4.92	1	0.85	0.8–0.9	0.06

All p-values after correcting for multiple testing; *df* degree of freedom; *OR* Odds ratio; *95% CI* 95 % confidence interval

withdrawal related seizures, and the number of DSM-IV alcohol-dependence symptoms (reflecting alcoholism severity). This present finding is contrary to the results of Schmidt et al. (2002), who reported a significant association between the same polymorphic site of CNR1 and a history of alcohol withdrawal delirium. No relationship between this polymorphism and a history of delirium tremens was detected in the present sample. Thus, this study fails to replicate the previous results of Schmidt et al. (2002).

Multiple testing between this genetic variant and alcoholism-related phenotypes, using Bonferroni adjustments, has been criticized by Perneger (1998) who identified several reasons not to perform Bonferroni adjustments for significance testing. One issue is the possible increase in type II errors so that truly important differences are deemed non-significant as the level of adjustment depends on the number of tests performed in the analysis.

However, even renouncing Bonferroni-correction for multiple testing, the weak relationship between a nonfunctional polymorphism and alcoholism withdrawal-related seizures detected in the current sample could very well be a spurious finding, common in association studies. Furthermore, it has been suggested that other multiple test procedures beyond the Bonferroni method be used to correct for multiple testing (Aikin 1999; Bender and Lange 1999). Taking these arguments together, the weak relationship between the nonfunctional genetic variant of CNR1 and alcohol-withdrawal induced seizures found in this study suggests that there is no association.

Since the actual relationship of a genetic variant and alcoholism-related phenotypes might be weak, most likely a number of genes are involved in creating a complex phenotype. Any genetic variant might have only a very small influence on the intensity of the phenotype. Furthermore, in a phenotype with a complex genetic background, a variety of other significant factors such as environment, culture and different levels of expression of several genes may be involved in creating a phenotype.

Thus, relationships between single genes of influence might be difficult to detect. Based on a sample size estimation performed based on Schmidt et al. (2002), the current study had sufficient power to detect a potential relationship.

Taking other genetic variants of CNR1 into account, other rare variants have been reported in the CNR1 gene of an epilepsy patient (Kathmann et al. 2000; Phe200Leu, Ile216Val, Val246Ala) which might not be appropriate candidates for subsequent haplotype analysis due to their low population frequencies.

However, the 5' regulatory region of CNR1, which is still under investigation, might be of importance for further genetic examination in relation to other alcoholism-related phenotypes.

In the present study, the genotyping was performed by the fluorescence resonance energy transfer method (FRET) in contrast to an allele-specific PCR in the study by Schmidt et al. (2002). Both methods are state of the art and widely used for genotyping studies. They are very robust against typing errors. Therefore, it is very unlikely that the methodological differences between our study and those of Schmidt et al. (2002) are responsible for the different findings.

In summary, our finding could not confirm the previous study of Schmidt et al. (2002) suggesting a potential involvement of a CB1 receptor genetic variant in alcohol withdrawal-related phenotypes. Other genetic, environmental, and cultural factors are likely to be involved in the etiology of alcoholism-related phenotypes such as alcohol-withdrawal-induced seizures.

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