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Increased Attributable Risk Related to a Functional μ-Opioid Receptor Gene Polymorphism in Association with Alcohol Dependence in Central Sweden

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The μ-opioid receptor (MOR), through its effects on reward and stress-responsivity, modulates alcohol intake in both animal and human laboratory studies. We have previously demonstrated that the frequently occurring A118G single-nucleotide polymorphism (SNP) in exon 1 of the MORgene (*OPRM1*), which encodes an amino-acid substitution, is functional and receptors encoded by the variant 118G allele bind the endogenous opioid peptide β-endorphin with three-fold greater affinity than prototype receptors. Other groups subsequently reported that this variant alters stress-responsivity in normal volunteers and also increases the therapeutic response to naltrexone (a μ-preferring opioid antagonist) in the treatment of alcohol dependence. We compared frequencies of genotypes containing an 118G allele in 389 alcohol-dependent individuals and 170 population-based controls without drug or alcohol abuse or dependence. The A118G SNP was present in the Hardy–Weinberg equilibrium with an overall frequency of the 118G allele of 10.9%. There was a significant overall association between genotypes with an 118G allele and alcohol dependence ($p = 0.0074$). The attributable risk for alcohol dependence in subjects with an 118G allele was 11.1%. There was no difference in A118G genotype between type 1 and type 2 alcoholics. In central Sweden, the functional variant 118G allele in exon 1 of *OPRM1* is associated with an increased attributable risk for alcohol dependence.

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INTRODUCTION

Twin registry and adoption studies have shown that the heritability of alcoholism may be as high as 50–60% (Kendler *et al*, 1997; Prescott and Kendler, 1999). While early genetic studies of alcoholism focused on archival and phenotypic data (eg Cloninger *et al*, 1981; Kaij, 1957), recent advances in molecular genetics have permitted a hypothesis-driven evaluation of specific genes, which animal and molecular studies have demonstrated are altered by alcohol. Examples of this latter approach include the study of genes encoding proteins involved in the metabolism of alcohol (eg alcohol dehydrogenase and acetaldehyde dehydrogenase); genes hypothesized to be associated with behaviors linked to an increased risk of alcoholism (eg impulsivity and tryptophan hydroxylase); and genes encoding or modulating the transcription of proteins involved in the reinforcing effects of alcohol (eg neurotransmitters).

The endogenous opioid system modulates diverse physiological functions such as stress responsivity, analgesia, cognition, gastrointestinal motility, and immune function (Kreek, 1996). Animal and human studies have shown that alcohol-nondependent offspring with an alcohol-dependent parent show altered responsivity of endogenous opioid mediated systems following alcohol consumption compared to offspring without a family history of alcohol dependence (Gianoulakis *et al*, 1996; Schuckit *et al*, 1987). In addition, pharmacological antagonism of μ-opioid receptors (MORs) reduces alcohol consumption in both animal models and human studies (Mason *et al*, 1994; Ulm *et al*, 1995; Volpicelli *et al*, 1992). Quantitative trait locus studies have identified the chromosomal region containing the MOR (*OPRM1*) gene as important to the development and perpetuation of alcohol consumption (Grisel, 2000). Also, knockout mice lacking *OPRM1* do not self-administer alcohol (Hall *et al*, 2001; Roberts *et al*, 2000).

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The *OPRM1* gene is therefore a logical candidate for genetic association studies of alcoholism. Several single-nucleotide polymorphisms (SNP) have been identified in this gene. Two of these, located within exon 1 (C17T and A118G), are common (ranging from 2 to 48% across populations), encode amino-acid substitutions, and have been studied by several investigators (Kreek *et al*, 2004; LaForge *et al*, 2000). The A118G SNP is of particular interest because, as we originally showed, it is a functional polymorphism resulting in three-fold increased binding affinity of the endogenous opioid β-endorphin and three-fold increased current across G protein-activated inwardly rectifying potassium channels following binding by β-endorphin (Bond *et al*, 1998). Subsequently, we hypothesized that one copy of the A118G variant might significantly alter physiological responses mediated by the MOR such as stress response (Bond *et al*, 1998; Kreek *et al*, 2004; LaForge *et al*, 2000). In separate studies, Wand *et al* (2002) and Hernandez-Avila *et al* (2003) showed that healthy volunteers with a variant 118G allele have increased hypothalamic-pituitary-adrenal (HPA) axis response following administration of the μ-preferring opioid antagonist naloxone. As acute alcohol administration and MOR antagonism each stimulate HPA axis activity (eg as measured by peripheral levels of ACTH and cortisol) and this activation is inversely correlated with alcohol craving, the 118G receptor variant may play an important role in the development or treatment of alcoholism through an HPA axis-mediated mechanism (O'Malley *et al*, 2002).

Association studies of the A118G polymorphism in the addictive diseases in general, and in alcoholism specifically, are, however, conflicting. Several studies have failed to find an association between either the A118 or the 118G allele and alcoholism (Bergen *et al*, 1997; Franke *et al*, 2001; Gelernter *et al*, 1999; Hoehe *et al*, 2000; Kim *et al*, 2004; Kranzler *et al*, 1998; Loh *et al*, 2004; Sander *et al*, 1998; Schinka *et al*, 2002), while others have found an association between the A118 allele and alcoholism (Town *et al*, 1999), and yet others have found that the 118G allele is associated with alcoholism (Luo *et al*, 2003). Possible explanations for these conflicting reports include, in some cases, small sample size of the populations under study and, in others, the ethnic heterogeneity of the ascertained populations and admixtures within any single ethnic/cultural group.

We recently reported a significant association between the 118G allele and heroin addiction in a population from central Sweden with up to 21% of the attributable risk for developing heroin addiction related to the presence of this polymorphism (Bart *et al*, 2004). In the present study of alcohol dependence, we again focused on a population from central Sweden in order to reduce the potential effect of population admixture.

METHODS

Patients and Controls

A total of 467 alcohol-dependent subjects were recruited from an academic addiction medicine clinic offering medically assisted detoxification and outpatient treatment. Diagnosis of alcohol dependence was determined through completion of the DSM-III-R Checklist by the admitting physician with subsequent verification from patient records by a research nurse. In total, 87 subjects were excluded from the final analysis for the following reasons: incomplete clinical information ($n=17$), met criteria for dependence on a drug in addition to alcohol ($n=47$), could not be successfully genotyped ($n=8$), sample contaminated ($n=1$), sample not genotyped ($n=14$). The final alcohol-dependent group consisted of 389 subjects (108 female, 281 male), most of whom were also subtyped into type 1 (late onset) and type 2 (early onset) alcoholism using revised criteria (Cloninger *et al*, 1981; Hallman *et al*, 1996). Individuals with major psychotic disorders were excluded from the study. Other Axis I and II diagnoses and familial history of alcoholism were not systematically assessed.

The control sample consisted of 170 healthy volunteers (88 female, 82 male) recruited as part of a large population-based general health survey. Subjects were in good health and did not meet any DSM-III-R SCID-I-defined criteria for any substance-related or psychotic disorder. Familial history of alcoholism was not systematically assessed.

Study subjects were primarily of Swedish ethnicity (Table 1). Subjects from central Sweden were chosen because of the relatively low frequency of population admixture in this region. This area experienced a large Finnish immigration in the 16th and 17th centuries. Some Baltic, central European, and southern European (primarily Italian) immigration occurred just before and following World War II. Until around 1970 when more diverse immigrant populations (American, Vietnamese, Iranian, Iraqi) began to arrive, the population of this catchment area had remained stable for several hundred years.

All subjects provided written consent for participation in genetic studies and the protocol was approved by the Stockholm South (patients) and Stockholm North (controls) Human Subjects Ethics Committee.

Genotyping of OPRM1

Genomic DNA was isolated from peripheral blood lymphocytes. For the control subjects, approximately 100 ng of genomic DNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers designed to amplify the complete coding region of exon 1 of *OPRM1*. Sequences of the primers were: 5′-CTC CGC CTG ACG CTC C-3′

Table 1 Ethnic Distribution

	Swedish	Finnish	Other European	African	Mideastern	Latino	Asian	Unknown/unspecified
Alcohol dependent ($n=389$)	300	72	9	2	0	1	0	5
Controls ($n=170$)	144	11	12	1	1	1	0	0

Ethnicity is self-identified.

(forward) and 5′-GTC CCC CCA GTT TAC CTC C-3′ (reverse). A step-down amplification was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a standard reaction volume of 50 μl. PCR products were electrophoresed on agarose gel to verify amplification and fragment size. PCR products were then purified and sequenced at The Rockefeller University DNA Sequencing Center using the following sequencing primers: 5′-AAA GGA AGC GGC TGA GGC-3′ (forward) and 5′-GTC CCC CCA GTT TAC CTC C-3′ (reverse). Genotypes were determined by two independent assessors (GB and KSL) who were blind to the diagnostic data.

Direct analysis of the A118G and C17T polymorphisms was performed in the alcohol-dependent subjects using fluorescent PCR in a TaqMan protocol using TaqMan MGB probes and primers designed according to Applied Biosystems specifications. This technique has been validated against the technique used for sequencing the control subjects.

Statistics

The overall frequency of each genotype and allele was determined. The odds ratio (OR) was calculated and the Mantel–Haenszel χ^2 test was performed to test the null hypothesis of no difference in genotype distribution between opiate-dependent and normal volunteers (Mantel and Haenszel, 1959). For 2×2 tables with rows corresponding to cases and controls, and columns corresponding to susceptible *vs* nonsusceptible genotypes, attributable risks were computed (Berry and Armitage, 1994), that is, the estimated proportion of cases in the population who are affected due to the given at-risk genotype(s).

RESULTS

In all, 389 individuals meeting the DSM-III-R criteria for alcohol dependence and 170 healthy controls with no drug or alcohol abuse or dependence were genotyped for the C17T and A118G polymorphisms in exon 1 of the *OPRM1* gene. The mean age (SD; range) of the alcohol-dependent subjects and controls was 55.8 (10.1; 29–85) and 45.6 (15.9; 21–75) years, respectively. Of these, 28% of the alcohol-dependent and 52% of control subjects were female. Genotype and allele distribution did not vary between genders, so gender stratification was not performed.

Since no subjects with the C17T polymorphism were identified, analysis focused on the A118G polymorphism. Distribution of the 118G allele was in the Hardy–Weinberg equilibrium with an overall allelic frequency of 10.9%. Analysis by genotype (autosomal dominant mode of inheritance) revealed significantly greater OR for alcohol dependence in subjects with an 118G allele (OR = 1.92, $\chi^2_{(1)} = 7.18$, $p = 0.0074$) (Table 2). The attributable risk due to genotypes with an 118G allele was 11.1% (95% CI 3.6–18.0%). There was no difference in A118G genotype between type 1 and type 2 alcoholics ($\chi^2_{(1)} = 0.01$, $p = 0.9$) (Table 2).

To minimize concerns about population admixture, a Mantel–Hanszel analysis comparing genotype data between Swedish subjects with two Swedish parents (193 alcohol dependent, 120 control) and all other subjects (196 alcohol dependent, 50 control) showed a significant association between genotypes with an 118G allele and alcoholism ($p = 0.0395$) (Table 3). Since genotype data for Swedish and non-Swedish individuals were not significantly different, for the above likelihood ratio and attributable risk statistics, the Swedish and non-Swedish subsets were pooled.

DISCUSSION

Our results support an association of the 118G allele in exon 1 of the MOR (*OPRM1*) gene to alcohol dependence, independent of type 1 and type 2 characteristics. In this study population from central Sweden, the attributable risk due to genotypes containing this allele is 11.1%.

Table 2 (a) Genotype Distribution for All Subjects; (b) Genotype Distribution by Cloninger Type 1 and Type 2 Alcohol Dependence

	Alcohol dependent ($n = 389$)	Control ($n = 170$)
(a) All subjects[a]		
A118	299	147
A118G, G118G	90	23
(b) Alcohol-dependent subjects[b]		
	Type 1 ($n = 242$)	**Type 2 ($n = 124$)**
A118	186	96
A118G, G118G	56	28

[a]χ^2 is computed in the form of the likelihood ratio statistic: $\chi^2_{(1)} = 7.18$, $p = 0.0074$.
[b]For 23 subjects, type 1 or type 2 classification is unknown; χ^2 is computed in the form of the likelihood ratio statistic: $\chi^2_{(1)} = 0.01$, $p = 0.904$.

Table 3 Genotype Distribution in Swedish Subjects with Two Swedish Parents *vs* All Other Subjects

	Swedish with two Swedish parents		Not Swedish with non Swedish parents	
	Alcohol dependent	**Control**	**Alcohol dependent**	**Control**
A118	158	104	141	43
A118G, G118G	35	16	55	7

Owing to the small numbers of G118G genotypes when individuals are split into a $2 \times 2 \times 3$ table (two ethnicities, two treatment groups, three genotypes), the G118G and A118G genotypes were pooled into an A-class (= presence of an 118G allele). A Mantel–Hanszel analysis was carried out for the stratified 2×2 tables, $p = 0.0395$, homogeneity 0.3511.

The endogenous opioid system is central to the development and persistence of the addictive diseases (Kreek, 1996). The MOR, whose principal endogenous ligand is β-endorphin, binds opiates as used in the treatment of pain (eg morphine) or abused illicitly (eg heroin and its active metabolites). It is involved in diverse physiological processes including mediation of the stress responsive hypothalamic–pituitary–adrenal (HPA) axis.

While glucocorticoids acting through negative feedback provide the major countermodulatory effect on the HPA axis, the MOR system exerts a secondary form of countermodulation through tonic inhibition (Kreek and Koob, 1998). Alterations in HPA responsivity following acutely and chronically administered opiates, cocaine, and alcohol have been demonstrated in rodent, non-human primate, and human studies (Kreek, 1992; Kreek *et al*, 2002). Short-acting opioids produce HPA axis suppression through direct effects on MOR, whereas alcohol and cocaine stimulate HPA axis activity through indirect opioidergic effects. Withdrawal from opioids and alcohol both activates the HPA axis and, in the case of opiate withdrawal, increased HPA axis activity precedes, and thus may contribute to the subjective effects of withdrawal (Culpepper-Morgan and Kreek, 1997).

Family history of alcoholism and thus presumably genetic factors may modulate the HPA-axis response to alcohol, but the nature of this modulation is unclear. Both blunted cortisol response (Schuckit *et al*, 1987) and enhanced ACTH and β-endorphin response to alcohol have been reported in nondependent offspring of alcoholic subjects (Gianoulakis *et al*, 1996). More consistently, increased HPA response to the μ-preferring opiate antagonists naloxone or naltrexone has been found in nonalcoholic offspring of alcoholics (King *et al*, 2002; King *et al*, 1997; Wand *et al*, 1998; Wand *et al*, 2001). Interestingly, naltrexone also attenuated the subjective effects of alcohol in the family history positive social drinkers but not in the family history negative group (King *et al*, 1997).

It is possible that alcohol is partly consumed for its ability to activate the HPA axis. Following a single priming drink, naltrexone-treated alcohol-dependent subjects drank less alcohol and experienced less craving than did placebo-treated subjects. Interestingly, naltrexone stimulated HPA axis to a similar extent as alcohol, and increased levels of plasma cortisol following naltrexone were correlated with decreased craving (O'Malley *et al*, 2002). While genetics were not part of this study, we hypothesized that the acute alcohol-mimetic effects of increased HPA activation following an opioid antagonist in subjects with the functional A118G SNP may produce a greater reduction in alcohol craving and therefore these subjects may be more responsive to opioid antagonist therapy. This hypothesis has recently received some support by the finding that alcohol-dependent subjects with an 118G allele return to heavy drinking following treatment with naltrexone later than do similarly treated alcoholics with the prototypic receptor (Oslin *et al*, 2003).

To date, association studies of alcohol dependence and the A118G SNP have found: (1) a significant association between a haplotype which included the 118G allele and mixed alcohol and opioid dependence (Luo *et al*, 2003); (2) association between the A118 allele and alcohol dependence (Town *et al*, 1999); and (3) no association between the A118G SNP and alcohol dependence (Bergen *et al*, 1997; Franke *et al*, 2001; Gelernter *et al*, 1999; Hoehe *et al*, 2000; Kranzler *et al*, 1998; Loh *et al*, 2004; Sander *et al*, 1998; Schinka *et al*, 2002). While Kim *et al* did not find an association between the A118G SNP and alcohol dependence in a Korean population, they did find that alcoholics homozygous for the 118G allele drank more days per month than alcoholics with a single 118G allele who drank more days per month than alcoholics without an 118G allele. Interestingly, there was no difference between genotypes in the amount of alcohol consumed per drinking day (Kim *et al*, 2004).

The conflicting results of the above studies may be due to differences in ethnicity of the populations under study, since in populations of different ethnic origins the frequency of the 118G allele can range from less than 2 to 48% (Bart *et al*, 2004; Bond *et al*, 1998; Gelernter *et al*, 1999; Li *et al*, 2000; Shi *et al*, 2002; Szeto *et al*, 2001; Tan *et al*, 2003). There may also be nongenetic-mediated environmental factors that contribute to the development of alcohol dependence to a differing extent within and between the ethnic groups previously studied. Here, we controlled for allelic frequency differences in populations by conducting the study in a predominantly Swedish population, which has experienced little admixture over the past several hundred years and remains fairly 'culturally homogeneous'. We also controlled for the type of alcoholism and found no difference in allele distribution between type 1 and type 2 alcoholics. While this control may increase the power of the current study, its generalizability to non-Swedish populations remains uncertain. Further studies replicating the current findings in Swedish and non-Swedish populations are needed.

Studying a population of Swedish heroin addicts, we recently found a significant association between the 118G allele and heroin addiction, compared to the same population-based control group used in the current study (Bart *et al*, 2004). In that report, the attributable risk for developing heroin addiction was 21% when subjects with two Swedish parents were analyzed and 18% for all subjects. In the present study, the attributable risk was more modest at 11% overall. This may indicate that the 118G allele contributes to a common susceptibility for developing addictive disorders, and additionally to the risk of developing opiate addiction. Alternatively, opioid mechanisms may be involved in development of alcohol dependence only in a subgroup of alcoholics, diluting the statistical effect size of the MOR polymorphism.

Surprisingly, we found no subjects with the C17T variant. The allelic frequency of 17T has ranged from 0.8 to 21% across ethnic groups (Berrettini *et al*, 1997; Bond *et al*, 1998; Gelernter *et al*, 1999). A trend ($p = 0.054$ or $p = 0.05$ Bond *et al* and Berrettini *et al*, respectively) towards a higher frequency of this variant and substance dependence has also been reported (Berrettini *et al*, 1997; Bond *et al*, 1998). As this variant, the second most frequently occurring SNP within exon 1 of *OPRM1*, was not identified, haplotype analysis incorporating this SNP could not be performed. This study may be limited by its focus on exon 1 of *OPRM1*, in that sequencing of the 5′ region as well as the introns and other exons of this gene would have allowed for haplotype

analyses. There may be, however, limited utility of haplotype analysis in this association study of the A118G SNP. By demonstrating the functional effect of the A118G SNP in a cellular construct and then in the human laboratory, it appears likely that this SNP is the locus of interest in this study, rather than a nearby coinherited allele. This does not exclude the undoubtedly oligogenetic nature of this complex disease or the need for further identification and study of other SNPs possibly coinherited with the 118G allele.

In conclusion, the current study found a significant association between genotypes with the 118G allele of this functional polymorphism in the MOR gene and alcohol dependence in central Sweden.

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