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The opioid peptides enkephalin and β -endorphin in alcohol dependence

Ildiko Racz^{*,1}, Britta Schürmann^{*,1}, Anna Karpushova², Martin Reuter³, Sven Cichon², Christian Montag³, Robert Fürst⁴, Christian Schütz⁵, Petra E. Franke^{5,7}, Jana Strohmaier⁷, Thomas F. Wienker⁴, Lars Terenius⁶, Urban Ösby⁶, Agneta Gunnar⁶, Wolfgang Maier⁵, Andras Bilkei-Gorzó¹, Markus Nöthen², and Andreas Zimmer¹

¹Institute of Molecular Psychiatry, University of Bonn, Germany

²Department of Medical Genetics, University of Bonn, Germany

³Differential Psychology Personality Research, University of Bonn, Germany

⁴Institut of Medical Biometry, Informatics and Epidemiology, University of Bonn, Germany

⁵Department of Psychiatry, University of Bonn, Germany

⁶Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden

⁷Central Institute of Mental Health, Mannheim, University of Heidelberg

⁸Department of Psychiatry, University of Düsseldorf

Abstract

Background—Experimental evidence indicates that the endogenous opioid system influences stress responses as well as reinforcing effects of addictive drugs. Because stress is an important factor contributing to drug dependence and relapse, we have now studied ethanol preference in enkephalin and β -endorphin deficient mice under baseline conditions and after stress exposure.

Methods—In the present study we used a two-bottle choice paradigm to study ethanol consumption, and stress-induced ethanol preference. To examine alcohol withdrawal symptoms the forced drinking procedure was employed. We performed an association analysis in two case-control samples of alcohol addicts to determine whether these opioid peptides also contribute to ethanol dependence in humans.

Results—Ethanol consumption was significantly reduced in the absence of β -endorphins, particularly in female knockout animals. Stress exposure results in an increased ethanol consumption in wild type mice, but did not influence ethanol drinking in β -endorphin knockouts. Enkephalin-deficient mice showed no difference to wild type mice in baseline ethanol preference, but also showed no stress-induced elevation of ethanol consumption. Interestingly, we found a two-marker haplotype in the *POMC* gene that was associated with alcohol dependence in females in both cohorts.

Conclusion—Together these results indicate a contribution of β -endorphin to ethanol consumption and dependence.

Corresponding author: Ildiko Racz, Institute of Molecular Psychiatry, Life & Brain Center, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany, Phone: +49-228-688 5316, Fax: +49-228-688 5301, email: iracz@uni-bonn.de; neuro@uni-bonn.de.
*equal contribution

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Keywords

alcohol; addiction; enkephalin; β -endorphin; case-control studies

INTRODUCTION

Genetic risk factors contribute to addiction disorders, including alcoholism (1,2). A recent survey has shown that nearly 100 genes have been studied for contribution to ethanol responses in genetically modified mice (3). These reports support pharmacological studies suggesting the involvement of various neurotransmitter systems as modulators of ethanol effects (glutamate, GABA, dopamine, serotonin, cannabinoids and opioids) (4).

The endogenous opioid system consists of μ -, δ -, and κ -opioid receptors (MOP, DOP and KOP) and the opioid peptides enkephalin, β -endorphin and dynorphin. These peptides are produced from specific precursorss (enkephalin/preproenkephalin [PENK], β -endorphin/proopiomelanocortin [POMC], dynorphin/preprodynorphin [PDYN]) (5). Enkephalins and β -endorphin are the endogenous ligands at MOP and DOP receptors. β -endorphin binds MOP and DOP receptors with approximately equal affinity, while enkephalin has a more than 10-fold higher affinity for the DOP receptor. Dynorphins selectively activate KOP receptors and additionally several non-opioid receptors (6).

Agonists for MOP and DOP receptors are rewarding for humans and animals. They stimulate the activity of the mesolimbic reward system and dopamine release in the nucleus accumbens. In contrast, KOP agonists can produce dysphoria and place aversion, and decrease dopamine levels through activation of presynaptic KOP receptors in the nucleus accumbens (7). Because ethanol exposure increased β -endorphin and enkephalin levels, the endogenous opioid system may mediate some of the rewarding effects of alcohol (8). In fact, pharmacological blockade of opioid receptors with unselective antagonists naloxone and naltrexone reduces ethanol consumption, similar to more selective DOP and MOP antagonists (9–11). Knockout mouse studies confirmed the role of MOP and DOP receptors in ethanol reward. Thus, MOP knockout mice show less ethanol-induced place preference and little or no ethanol self-administration, with a stronger effect in females compared to males. In contrast DOP knockouts drank more alcohol than wild type (WT) mice (12–14). Surprisingly, these results were so far not supported by the analysis of mice with a genetic deletion of enkephalin (ENK-KO) or β -endorphin (END-KO). Thus, ENK-KO mice showed a similar operant ethanol self-administration as WT animals, while END-KO mice displayed a somewhat increased ethanol self-administration or preference. Even double knockout mice lacking both enkephalin and β -endorphin learned to self-administer ethanol similarly to WT animals (15). As the deletion of both of these peptides should result in a much-reduced MOP tone, it is difficult to understand why the peptide and the receptor knockout phenotypes do not match.

A number of studies in humans have evaluated the role of polymorphisms in opioid receptors in samples of alcoholic patients from diverse ethnic origin. Many of those have focused on a MOP coding variant, A118G. The results from these studies remain, by and large, inconclusive, because positive and negative results have been reported (16–29). Whereas two coding variants of the DOP receptor, G80T and T921C, were analysed in alcohol-dependent Taiwanese Hans (21) and heroin- and alcohol-dependent Caucasians of German origin (30). None of these two studies found evidence for an association. One study has analyzed a CA repeat at the 3' end of *PENK*, but found no association with alcohol dependence in populations of Asian, African-American, and Caucasian origin (31). A recent analysis of SNPs in *POMC* and *PENK* in multiplex alcohol dependent Caucasian American families also provided no support for the association of *PENK* and *POMC* polymorphisms with alcohol dependence. However, allelic

variants in the *PENK* and *POMC* gene were associated with the narrower phenotype of opioid dependence in these families (29).

In the present study we first evaluate END-KO and ENK-KO mice in paradigms for ethanol preference, somatic dependence and stress-induced alcohol drinking, followed by genetic analyses of cohorts of alcoholic patients. Because previous studies have demonstrated a gender specific effect of *Oprm1* deletion on ethanol preference, we analyzed males and females separately. Our results provide evidence for a sex-specific role of β -endorphin in ethanol preference and dependence.

METHODS AND MATERIALS

Animals

The generation of enkephalin-deficient and β -endorphin deficient mice has been described previously (32,33). The targeted mutation of the preproenkephalin *Penk1* locus completely disrupted the gene, thus resulting in a null allele. In contrast, β -endorphin deficient mice were generated by introducing a stop codon just upstream of the β -endorphin peptide sequence, consequently leaving all other POMC peptides intact (34). All animals were crossed for more than 10 generations to C57BL/6J mice and were therefore congenic for this genetic background. Here we refer to preproenkephalin knockout mice as ENK-KO and to β -endorphin knockout animals as END-KO.

Animals were housed individually under reversed light-dark conditions (lights on – 7 pm, lights off – 9 am) with free access to food and drinking solutions. Experiments were conducted with 8–10 week old mice.

Acute alcohol effect and tolerance

To determine ethanol-induced hypothermia, we first measured rectal body temperature of ethanol-naïve mice - using a stainless steel rectal probe designed for mice, and connected to a portable thermocouple thermometer (BAT-12, Harvard Instruments, USA). This was immediately followed by an intraperitoneal injection of ethanol (saline, 1 g/kg, 2 g/kg and 4 g/kg). Body temperature was determined again 30 minutes after the injection.

Ethanol-induced hypothermia was expressed as the difference in body temperature before and after the injection. In order to determine the development of alcohol tolerance to the hypothermic effects of ethanol we performed the same experiment after the animals run through a forced drinking period of 22 days. In this period animals obtained an ethanol solution as their only fluid source. The ethanol concentration was gradually increased as follows: day 1–3, 4% ethanol; day 4–6, 8% ethanol; and from day 7 onwards 16% ethanol. Tolerance was assessed as a decrease in the hypothermic effect of acute alcohol treatment following the forced drinking procedure.

Alcohol preference and stress-induced ethanol drinking

Ethanol preference was assessed as previously described (35,36). Briefly, two drinking bottles with 8% v/v alcohol (EtOH) or drinking water were available to the animals ad libitum. The positions of the bottles were changed daily. The ratio of alcohol to total fluid consumption, the amount of consumed ethanol (g/kg), the body weight (g), and food consumption (g) were determined twice a week. These data were used to calculate the average daily consumption.

Animals that have been studied in the two bottle choice paradigm for two months were then exposed a mild foot shock (see supplementary material). Animals were then returned to their home cages with ethanol and water bottles. To assess the effect of stress on ethanol preference

alcohol and water consumption were determined 24 h and 96 h after the shock, and calculated as the average daily consumption.

Physical signs of withdrawal

To determine physical signs of dependence animals received 16% ethanol solution in the forced drinking procedure as described above. After four weeks it was replaced with water. Scoring of handling induced convulsion (HIC-score) was performed at first in ethanol naïve animals, next at the end of the drinking phase to obtain a baseline as well as three hours after withdrawal as previously described (37). The animals were lifted by the tail and held for 3 s, rotated gently and their behaviour was scored on a scale between zero and three as follows: 0 = no tremor or convulsion, 1 = mild tremor on lifting and turning, 2 = continuous severe tremor on lifting and turning, 3 = clonic forelimb extensor spasm on lifting. All scorings were performed in a blinded manner by the same investigator.

Human subjects

In our exploratory study the group comprised 247 patients with a definite current DSM-IV diagnosis of alcohol dependence (180 men, mean age = 42.0 ± 9.0 ; 67 women, mean age = 44.3 ± 8.5) and 247 population-based control subjects (180 men, mean age = 41.7 ± 8.9 ; 67 women, mean age = 44.2 ± 8.4). The sample size of the female subgroup is different for marker rs934778 and rs3769671 (patients: 83 women, mean age = 44.4 ± 8.6 ; controls: 140 women, mean age 45.8 ± 9.6). Substance abuse and dependence (including alcohol and illicit drugs) as well as psychiatric disorders were assessed using the whole Structured Clinical Interview for DSM-IV Axis I Disorders (SCID). An experienced psychiatrist made the DSM-IV diagnosis of alcoholism based on the SCID-interview and all available clinical information and records. Subjects that additionally met DSM-IV criteria for dependence on an illicit drug or had recently (one year) any major psychiatric disorder were excluded. A history of psychiatric disorders, such as a history of a depressive episode did not lead to exclusion. Patients were recruited at the University of Bonn, Department of Psychiatry, or collaborating psychiatric hospitals in Düsseldorf, Mainz and Essen. The population-based control sample of Caucasians of German origin was collected with the support of the local Census Bureau of the city Bonn. The sample was established within the framework of the German National Genome Research Network I (NGFN I; www.ngfn.de) of the Federal Ministry of Education and Research between the years 2000 and 2003 to serve as an epidemiological control sample for complex genetic studies within the NGFN. To further verify our findings, a group of healthy control women consisted of German blood donors (120 individuals, mean age 29.5 ± 10.1) and a group of female alcoholics ($n = 72$; mean age = 47.3 ± 10.6) were genotyped for marker rs934778 and rs3769671. The female patients were recruited in the Clinic Eschenburg, a hospital specializing in treatment of alcohol abuse and alcoholism. They met ICD-10 criteria for alcohol dependence (F10.25) (International Classification of Diseases tenth revision (ICD-10) of the World Health Organization (WHO)) and came to the rehabilitation clinic after alcohol detoxification and participated in the study directly after admission to the Clinic Eschenburg. The individuals in this group had no clinical diagnosis of dependence on an illicit drug. All patients and controls were Caucasians of German origin.

In our replication study affected individuals (240 males mean age = 47.25 ± 10.65 ; 114 females, mean age 46.09 ± 10.39) and controls (202 males, 113 females) of Scandinavian ancestry served as a replication cohort. The alcoholic patients were collected within the Swedish Alcoholism in Siblings Study. Alcoholism was defined according to DSM-IV criteria after a structural psychiatric interview with the Schedules for Clinical Assessment in Neuropsychiatry (38). The co-morbidity with any other psychiatric disorder was less than 1%. A group of healthy control individuals consisted of Swedish blood donors. There was no information available concerning the age of the controls.

SNP selection, genotyping and statistical analysis

According to HapMap project data for Caucasians the human *PENK* gene is located in a single 92 kb haplotype block. The predicted tagging SNPs for this block were analysed. In contrast markers of the human *POMC* gene locus show weak linkage disequilibrium. We therefore randomly selected SNPs with an average spacing of 3 kb. DNA was isolated from whole blood or permanent cell lines derived from Epstein–Barr virus-transformed lymphocytes. MALDI-TOF mass spectrometry-based SNP genotyping was performed on a MassARRAY platform using iPLEX chemistry (Sequenom, Inc., San Diego, CA, <http://www.sequenom.com/>). The genotyping success rates were 98 % or higher. Additional probes were genotyped using the ABI Assays-On-Demand™ SNP Genotyping products. Statistical analysis was performed using the program FAMHAP version 17 (39–41). The option *singlecc* was used to calculate unadjusted p-values for association of single markers. For haplotype analysis FAMHAP estimates haplotype frequencies with the EM-algorithm (39). Using the option *hapcc*, p-values were computed by a permutational chi-square based test.

The statistical analysis of the animal experiments is described in supplemental material and methods.

RESULTS

Acute alcohol effect and tolerance

As shown in Table 1, alcohol treatment of drug naïve WT, ENK-KO and END-KO mice caused dose-dependent hypothermia, with significant effects at a dose of 2 g/kg and 4 g/kg ethanol ($F [3,168] = 272.09$, $p < 0.001$). Wt and ENK-KO animals developed tolerance to alcohol after one-month of ethanol drinking, as only 4 g/kg, but not 2 g/kg induced significant hypothermia ($F [1,168] = 5.107$, $p < 0.025$). On the other hand, END-KO mice developed no tolerance during the forced drinking regiment. We did not find gender-specific differences.

Alcohol preference and stress-induced ethanol drinking

To examine ethanol consumption, the animals had a free choice between an alcohol solution and water. The preference and the amount of ethanol consumed were correlated ($r = 0.85–0.9$) in WT and ENK-KO animals. In END-KO mice there were no correlations but preference and ethanol consumption pointed to the same direction (data not shown).

Because, female mice generally showed a higher preference for EtOH than males ($F [1,216]$ $p < 0.0001$), we examined the two genders separately (Fig. 1.). END-KO female animals consumed significantly less EtOH, and showed significantly lower preference for alcohol compared to WT and ENK-KO females ($F [2,822]$ $p < 0.0001$), whereas in the two latter groups we found no difference ($F [1,588]$ $p = 0.77$) (Fig.1.). EtOH consumption and preference remained stable during the seven weeks testing period.

Ethanol consumption was also the lowest in male END-KO mice compared to WT and ENK-KO males ($F [2,894]$ $p < 0.0001$), although END-KO and WT animals showed the same preference for alcohol. Thus, the combined liquid consumption was higher in WT compared to END-KO mice. We found no significant difference between Wt and ENK-KO animals in EtOH consumption ($F [1,678]$ $p = 0.14$) (Fig. 1.), but in the first three weeks the ENK-KO mice showed significantly higher preference for EtOH. All strains showed a small, but significant decrease of EtOH consumption during the examined period ($F [6,894]$ $p < 0.0001$), but this effect was more pronounced in the ENK-KO animals.

In order to assess the effect of stress on ethanol preference, we exposed the animals to a foot shock. We routinely use only male mice for these experiments, because female C57BL/6J

already have a high EtOH preference that is not further elevated after a foot shock. Stress caused a significant elevation in EtOH consumption of WT mice 24 h after the exposition (F [3,211] $p = 0.03$). This effect was transient and normalized after 96 h (Fig. 2a, 2b). In contrast, ENK-KO animals consumed significantly less EtOH 24 h and 96 h after foot shock (F [3,115] $p = 0.025$). Thus the stressor produced not an increase, but a paradoxical decrease in EtOH consumption (Fig 2a). We found no effect of stress on alcohol preference in END-KO animals (F [3,116] $p = 0.46$) (Fig. 2b).

Physical signs of ethanol dependence

Physical signs of dependence were analyzed by evaluation of HIC-scores in ethanol naïve animals, at the end of EtOH drinking phase and three hours after withdrawal from alcohol. HIC-scores showed no gender difference, thus we combined the results from both sexes. In all examined strains there was a clear increase in HIC-scores after withdrawal ($p < 0.001$). In END-KO mice we found a slight, but not significant elevation of HIC-scores in the EtOH-drinking group (Table 2.).

SNP genotyping

Having established that deficiency of enkephalin and endorphin affects behaviours relevant to ethanol dependence in mice, we hypothesized that these genes also contribute to ethanol dependence in humans. We therefore performed a case-control study in a German sample of alcohol dependent patients and controls, followed by a replication study in a Swedish cohort. We genotyped all five predicted tagging SNPs covering a haplotype block of 92 kb (HapMap) around the human *PENK* gene encompassing the two exons. We had to exclude marker rs10108778 due to assay problems. SNP rs12545109 is located 40 kb downstream the 3' end and rs2670029 26 kb upstream of the transcription initiation site (Table 3; Figure 3). The indicated haplotype block for markers rs2576581, rs12548084 and rs2670029 based on the LD-structure of German controls (Figure 3) is part of the corresponding haplotype block predicted by HapMap data for Caucasian populations. In contrast, markers at the *POMC* gene locus showed weak linkage disequilibrium in Caucasians (HapMap data). We therefore selected 9 SNPs with an even spacing of approximately 3 kb extending from 11 kb upstream of exon 1 up to 6 kb downstream exon 3 in German controls (Figure 3). In accordance with data from HapMap, we also found weak LD between markers.

Because we observed sex specific phenotype differences in our knockout mice we evaluated the allele and genotype distributions in the entire samples as well as in the female and male subgroups. In the German sample, all markers showed expected allele frequencies (Table 3) and revealed no significant deviations from Hardy-Weinberg-equilibrium (HWE) ($p > 0.05$). The unadjusted p-values for the *PENK* locus for allele frequencies of rs12545109 ($p = 0.031$) and rs2576581 in females ($p = 0.007$) (Table 3) suggested association with the disease phenotype. Both SNPs also showed low p-values for genotype distributions between the overall group of alcoholic patients and controls (Table 4). After *Bonferroni* correction for multiple testing only marker rs2576581 remained below the 5% significance threshold (allele distribution corrected $p = 0.028$; *Armitage's Trend Test* corrected $p = 0.036$). However, we were not able to confirm this in the Swedish replication sample (Table S1; Table S2).

For rs2164808 and rs934778, located upstream of the *POMC* gene and in intron 1 respectively, we found differences for allele and genotype distributions between German female alcoholic patients and controls (Table 3 and 4). In the Swedish replication sample, we found evidence for an association of marker rs934778 in the entire sample and of marker rs874401 in females (Table S1 and S2). However after *Bonferroni* correction, only marker rs934778 showed significance in German females (allele distribution corrected $p = 0.036$; *Armitage's Trend Test* corrected $p = 0.027$).

We next performed a haplotype analysis using sliding windows and found no PENK haplotypes with significant frequency differences between cases and controls (data not shown). Interestingly, for POMC we detected an association with the T-A haplotype of marker rs934778 and rs3769671 in the group of alcohol dependent females in both the German ($p = 0.012$) and the Swedish ($p = 0.016$) samples (Table 5).

DISCUSSION

Endogenous opioid peptides modulate the effects of many drugs of abuse. Here we have analyzed the role of enkephalins and β -endorphin in animal models of ethanol preference, ethanol preference after stress exposure, ethanol withdrawal symptoms, and in a human case-control study with alcoholic patients. Our results indicate that β -endorphin modulates ethanol preference and stress-induced relapse in animals. In humans, polymorphisms in the β -endorphin encoding POMC gene are associated with alcoholism. Interestingly, we found sex-specific differences in animal and in human studies.

Our findings are entirely consistent with the previous demonstration that voluntary ethanol intake was decreased in MOP knockout animals (12,13,42), i.e. in animals lacking the receptor with the highest affinity for β -endorphin. They also agree with the phenotype of DOP knockouts, which showed increased ethanol consumption. Together these data suggest that endorphin/MOP and enkephalin/DOP signalling modulates ethanol preference and consumption in mice. This is indeed one of the few examples showing a perfect match of receptor/ligand knockout phenotypes, thus lending further confidence to the relevance of these findings. Although enkephalins can also bind and activate MOP, it is unlikely that they contribute much to the MOP-mediated modulation of ethanol reward, because we and others found no difference between WT and ENK-KO mice in a two-bottle-choice and a conditioned place-preference paradigm (43). Together these findings suggest that β -endorphin acting on MOP plays a critical role in ethanol reinforcement, whereas both opioid peptides seem to be involved in stress-induced relapse.

Our results do not agree with previous studies reporting on operant intravenous and oral self-administration of ethanol, as well as ethanol consumption in a two bottle-choice paradigm in β -endorphin deficient mice. We show here that mice lacking β -endorphin consumed less of an 8% ethanol solution than wildtype controls. In contrast Grisel and co workers found a higher ethanol preference in β -endorphin knockout mice compared to wildtype animals with an ethanol concentration of 7%, but not with 10% (44). A study by Grahame and colleagues (45) supported these findings, because they found no significant genotype effect in a free access paradigm with an ethanol concentration of 10%, but increased ethanol consumption in END-deficient mice in a scheduled access paradigm where EtOH was only available for 2 hours per day. They suggested that consumption of 10% alcohol during limited access is opioid dependent, because naltrexone treatment reduced drinking in wild type and in knockout mice (46). In a paradigm for intravenous operant self-administration, END-deficient mice readily acquired self-administration behaviours, while wild-type mice did not. No genotype effects were observed when ethanol was delivered orally in the operant paradigm (45). The reason for these disparate results is not clear. Because the genetic background of the animals was very similar in all studies (C57BL/6), procedural or environmental effects are most likely to account for these differences. One obvious environmental factor is the ambient stress level to which the animals are exposed. Environmental stressors include odorants, noise, vibrations and other factors that differ between animal facilities. Indeed, we found a significant genotype effect after stress exposure, which increased EtOH consumption in wild type, but not in END-KO mice. Endogenous opioids and their receptors are expressed in neuronal stress-response circuits including the paraventricular nucleus (PVN), the nucleus of the solitary tract and raphe nuclei (47–50), which are also involved in the modulation drug reward (51). It is well known that

stress increases ethanol consumption in mice (36) and contributes to ethanol-seeking behavior in humans (52). Thus endogenous opioids may modulate ethanol consumption in stress conditions.

Because several previous studies have shown that female mice have a higher ethanol preference than males, we analyzed both sexes independently. We found that the difference in ethanol consumption between WT and END-KO mice was much larger in females than in males. Thus, the lack of β -endorphin affected female mice more strongly than males.

Important gender differences have also been observed for substance abuse disorders, including alcoholism, in humans (53). These differences relate to the age of onset, motivation for drug use, co-morbidities, and numerous other factors. For example, in heavy drinkers adrenocorticotrophic hormone and pituitary β -endorphin were significantly lower compared to controls, while plasma cortisol level were higher. These differences in hormone levels were more pronounced in female than in male subjects. It was therefore interesting to find that the *POMC* two-marker haplotype (rs934778, rs3769671) was associated with alcoholism in females, but not in males. Because the female subgroup was relatively small, the effect size (odds ratio) of the polymorphism may have been overestimated. Nevertheless, we found the same association in the Swedish replication sample. In contrast, the association of markers rs2576581 and rs12545109 (*PENK*) with the alcohol dependence phenotype in German females was not replicated in the Swedish cohort. Together our findings in humans and animals strongly support a contribution of β -endorphin to ethanol dependence in females. As previous studies in MOP receptor knockouts have also shown a stronger effect in females (12), our finding further demonstrates a gender bias of the endogenous opioid system in alcohol dependence. Although alcohol consumption is higher in men compared to woman, this is probably culturally biased. Nevertheless, our results further contribute to the idea that the genetic regulation of drug reward and dependence differs between genders.

A recent study using a diverse cohort of European Americans from alcohol dependent families found no association between *PENK* or *POMC* polymorphisms and alcoholism (29). For *PENK*, the study used different sets of markers. For *POMC*, Xuei et al. also analyzed rs934778 (SNP of the haplotype linked with alcoholism in our sample), but did not find a significant association of this SNP with alcohol dependence. Nevertheless, this marker showed a significant association with a more severe subtype of alcoholism, which is characterized by co-morbidity with opioid dependence (29). Unfortunately, Xuei et al did not differentiate between female and male gender in their sample, which encompassed predominantly males.

Taken together these data suggest, a contribution of endogenous opioid system to behaviours associated with alcoholism. β -endorphin seems to be important for ethanol preference, and enkephalin and β -endorphin modulate the effects of stress on ethanol consumption. A significant role of *POMC* in females was confirmed in our human association studies. Thus, the correlation of distinct alcohol related behaviours with the endogenous opioid peptides deserves further studies in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Reich T, Hinrichs A, Culverhouse R, Bierut L. Genetic studies of alcoholism and substance dependence. *Am J Hum Genet* 1999 Sep;65(3):599–605. [PubMed: 10441565]
2. Crabbe JC. Alcohol and genetics: new models. *Am J Med Genet* 2002 Dec 8;114(8):969–974. [PubMed: 12457395]
3. Crabbe JC, Metten P, Ponomarev I, Prescott CA, Wahlsten D. Effects of genetic and procedural variation on measurement of alcohol sensitivity in mouse inbred strains. *Behav Genet* 2006 Jul;36(4):536–552. [PubMed: 16619134]
4. Chastain G. Alcohol, neurotransmitter systems, and behavior. *J Gen Psychol* 2006 Oct;133(4):329–335. [PubMed: 17128954]
5. Kieffer BL, Gaveriaux-Ruff C. Exploring the opioid system by gene knockout. *Prog Neurobiol* 2002 Apr;66(5):285–306. [PubMed: 12015197]
6. Clarke S, Zimmer A, Zimmer AM, Hill RG, Kitchen I. Region selective up-regulation of micro-, delta- and kappa-opioid receptors but not opioid receptor-like 1 receptors in the brains of enkephalin and dynorphin knockout mice. *Neuroscience* 2003;122(2):479–489. [PubMed: 14614912]
7. Koob GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* 1992 May;13(5):177–184. [PubMed: 1604710]
8. Gianoulakis C. Endogenous opioids and addiction to alcohol and other drugs of abuse. *Curr Top Med Chem* 2004;4(1):39–50. [PubMed: 14754375]
9. Coonfield DL, Hill KG, Kaczmarek HJ, Ferraro FM 3rd, Kiefer SW. Low doses of naltrexone reduce palatability and consumption of ethanol in outbred rats. *Alcohol* 2002 Jan;26(1):43–47. [PubMed: 11958946]
10. Middaugh LD, Kelley BM, Bandy AL, McGroarty KK. Ethanol consumption by C57BL/6 mice: influence of gender and procedural variables. *Alcohol* 1999 Apr;17(3):175–183. [PubMed: 10231165]
11. Mitchell JM, Tavares VC, Fields HL, D'Esposito M, Boettiger CA. Endogenous opioid blockade and impulsive responding in alcoholics and healthy controls. *Neuropsychopharmacology* 2007 Feb;32(2):439–449. [PubMed: 17047667]
12. Hall FS, Sora I, Uhl GR. Ethanol consumption and reward are decreased in mu-opiate receptor knockout mice. *Psychopharmacology (Berl)* 2001 Feb;154(1):43–49. [PubMed: 11292005]
13. Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, et al. mu-Opioid receptor knockout mice do not self-administer alcohol. *J Pharmacol Exp Ther* 2000 Jun;293(3):1002–1008. [PubMed: 10869404]
14. Roberts AJ, Gold LH, Polis I, McDonald JS, Filliol D, Kieffer BL, et al. Increased ethanol self-administration in delta-opioid receptor knockout mice. *Alcohol Clin Exp Res* 2001 Sep;25(9):1249–1256. [PubMed: 11584142]
15. Hayward MD, Hansen ST, Pintar JE, Low MJ. Operant self-administration of ethanol in C57BL/6 mice lacking beta-endorphin and enkephalin. *Pharmacol Biochem Behav* 2004 Sep;79(1):171–181. [PubMed: 15388297]
16. Bergen AW, Kokoszka J, Peterson R, Long JC, Virkkunen M, Linnoila M, et al. Mu opioid receptor gene variants: lack of association with alcohol dependence. *Mol Psychiatry* 1997 Oct-Nov;2(6):490–494. [PubMed: 9399694]
17. Sander T, Gscheidel N, Wendel B, Samochowiec J, Smolka M, Rommelspacher H, et al. Human mu-opioid receptor variation and alcohol dependence. *Alcohol Clin Exp Res* 1998 Dec;22(9):2108–2110. [PubMed: 9884158]

18. Gelernter J, Kranzler H, Cubells J. Genetics of two mu opioid receptor gene (OPRM1) exon I polymorphisms: population studies, and allele frequencies in alcohol- and drug-dependent subjects. *Mol Psychiatry* 1999 Sep;4(5):476–483. [PubMed: 10523821]
19. Gscheidel N, Sander T, Wendel B, Heere P, Schmidt LG, Rommelspacher H, et al. Five exon 1 variants of mu opioid receptor and vulnerability to alcohol dependence. *Pol J Pharmacol* 2000 Jan–Feb;52(1):27–31. [PubMed: 10949117]
20. Franke P, Wang T, Nothen MM, Knapp M, Neidt H, Albrecht S, et al. Nonreplication of association between mu-opioid-receptor gene (OPRM1) A118G polymorphism and substance dependence. *Am J Med Genet* 2001 Jan 8;105(1):114–119. [PubMed: 11424981]
21. Loh el W, Fann CS, Chang YT, Chang CJ, Cheng AT. Endogenous opioid receptor genes and alcohol dependence among Taiwanese Han. *Alcohol Clin Exp Res* 2004 Jan;28(1):15–9. [PubMed: 14745298]
22. Town T, Abdullah L, Crawford F, Schinka J, Ordorica PI, Francis E, et al. Association of a functional mu-opioid receptor allele (+118A) with alcohol dependency. *Am J Med Genet* 1999 Oct 15;88(5):458–461. [PubMed: 10490697]
23. Schinka JA, Town T, Abdullah L, Crawford FC, Ordorica PI, Francis E, et al. A functional polymorphism within the mu-opioid receptor gene and risk for abuse of alcohol and other substances. *Mol Psychiatry* 2002;7(2):224–228. [PubMed: 11840318]
24. Luo X, Kranzler HR, Zhao H, Gelernter J. Haplotypes at the OPRM1 locus are associated with susceptibility to substance dependence in European-Americans. *Am J MedGenet B Neuropsychiatr Genet* 2003 Jul 1;120(1):97–108.
25. Kim SG, Kim CM, Kang DH, Kim YJ, Byun WT, Kim SY, et al. Association of functional opioid receptor genotypes with alcohol dependence in Koreans. *Alcohol Clin Exp Res* 2004 Jul;28(7):986–990. [PubMed: 15252283]
26. Bart G, Kreek MJ, Ott J, LaForge KS, Proudnikov D, Pollak L, et al. Increased attributable risk related to a functional mu-opioid receptor gene polymorphism in association with alcohol dependence in central Sweden. *Neuropsychopharmacology* 2005 Feb;30(2):417–422. [PubMed: 15525999]
27. Nishizawa D, Han W, Hasegawa J, Ishida T, Numata Y, Sato T, et al. Association of micro-opioid receptor gene polymorphism A118G with alcohol dependence in a Japanese population. *Neuropsychobiology* 2006;53(3):137–141. [PubMed: 16679777]
28. Zhang H, Luo X, Kranzler HR, Lappalainen J, Yang BZ, Krupitsky E, et al. Association between two {micro}-opioid receptor gene (OPRM1) haplotype blocks and drug or alcohol dependence. *Hum Mol Genet* 2006 Mar 15;15(6):807–819. [PubMed: 16476706]
29. Xuei X, Flury-Wetherill L, Bierut L, Dick D, Nurnberger J Jr, Foroud T, et al. The opioid system in alcohol and drug dependence: Family-based association study. *Am J Med Genet B Neuropsychiatr Genet.* 2007 May 14;
30. Franke P, Nothen MM, Wang T, Neidt H, Knapp M, Lichtermann D, et al. Human delta-opioid receptor gene and susceptibility to heroin and alcohol dependence. *J Med Genet* 1999 Oct 15;88(5):462–464.
31. Chan RJ, McBride AW, Thomasson HR, Ykenney A, Crabb DW. Allele frequencies of the preproenkephalin A (PENK) gene CA repeat in Asians, African-Americans, and Caucasians: lack of evidence for different allele frequencies in alcoholics. *Alcohol Clin Exp Res* 1994 Jun;18(3):533–535. [PubMed: 7943650]
32. Konig M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, et al. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 1996 Oct 10;383(6600):535–538. [PubMed: 8849726]
33. Bilkei-Gorzo A, Racz I, Michel K, Zimmer A, Klingmuller D, Zimmer A. Behavioral phenotype of pre-proenkephalin-deficient mice on diverse congenic backgrounds. *Psychopharmacology (Berl)* 2004;176(3–4):343–352. [PubMed: 15197532]
34. Rubinstein M, Mogil JS, Japon M, Chan EC, Allen RG, Low MJ. Absence of opioid stress-induced analgesia in mice lacking beta-endorphin by site-directed mutagenesis. *Proc Natl Acad Sci U S A* 1996 Apr 30;93(9):3995–4000. [PubMed: 8633004]

35. Little HJ, O'Callaghan MJ, Butterworth AR, Wilson J, Cole J, Watson WP. Low alcohol preference among the "high alcohol preference" C57 strain of mice; preference increased by saline injections. *Psychopharmacology (Berl)* 1999 Nov;147(2):182–189. [PubMed: 10591886]
36. Racz I, Bilkei-Gorzo A, Toth ZE, Michel K, Palkovits M, Zimmer A. A critical role for the cannabinoid CB1 receptors in alcohol dependence and stress-stimulated ethanol drinking. *J Neurosci* 2003 Mar 15;23(6):2453–2458. [PubMed: 12657705]
37. Watson WP, Little HJ. Correlation between increases in dihydropyridine binding in vivo and behavioural signs of ethanol withdrawal in mice. *Alcohol Alcohol* 1999 Jan–Feb;34(1):35–42. [PubMed: 10075399]
38. Wing JK, Babor T, Brugha T, Burke J, Cooper JE, Giel R, et al. SCAN. Schedules for Clinical Assessment in Neuropsychiatry. *Arch Gen Psychiatry* 1990 Jun;47(6):589–593. [PubMed: 2190539]
39. Becker T, Knapp M. Maximum-likelihood estimation of haplotype frequencies in nuclear families. *Genet Epidemiol* 2005 Jul;27(1):21–32. [PubMed: 15185400]
40. Becker T, Schumacher J, Cichon S, Baur MP, Knapp M. Haplotype interaction analysis of unlinked regions. *Genet Epidemiol* 2005 Dec;29(4):313–322. [PubMed: 16240441]
41. Becker T, Cichon S, Jonson E, Knapp M. Multiple testing in the context of haplotype analysis revisited: application to case-control data. *Ann Hum Genet* 2005 Nov;69(Pt 6):747–756. [PubMed: 16266412]
42. Becker A, Grecksch G, Kraus J, Loh HH, Schroeder H, Holtt V. Rewarding effects of ethanol and cocaine in mu opioid receptor-deficient mice. *Naunyn Schmiedeberg's Arch Pharmacol* 2002 Apr;365(4):296–302. [PubMed: 11919654]
43. Koenig HN, Olive MF. Ethanol consumption patterns and conditioned place preference in mice lacking preproenkephalin. *Neurosci Lett* 2002 Jun 7;325(2):75–78. [PubMed: 12044625]
44. Grisell JE, Mogil JS, Grahame NJ, Rubinstein M, Belknap JK, Crabbe JC, et al. Ethanol oral self-administration is increased in mutant mice with decreased beta-endorphin expression. *Brain Res* 1999 Jul 17;835(1):62–67. [PubMed: 10448196]
45. Grahame NJ, Low MJ, Cunningham CL. Intravenous self-administration of ethanol in beta-endorphin-deficient mice. *Alcohol Clin Exp Res* 1998 Aug;22(5):1093–1098. [PubMed: 9726280]
46. Grahame NJ, Mosemiller AK, Low MJ, Froehlich JC. Naltrexone and alcohol drinking in mice lacking beta-endorphin by site-directed mutagenesis. *Pharmacol Biochem Behav* 2000 Dec;67(4):759–766. [PubMed: 11166066]
47. Williams RG, Dockray GJ. Distribution of enkephalin-related peptides in rat brain: immunohistochemical studies using antisera to met-enkephalin and met-enkephalin Arg6Phe7. *Neuroscience* 1983 Jul;9(3):563–586. [PubMed: 6312371]
48. Fallon JH, Leslie FM. Distribution of dynorphin and enkephalin peptides in the rat brain. *J Comp Neurol* 1986 Jul 15;249(3):293–336. [PubMed: 2874159]
49. Finley JC, Lindstrom P, Petrusz P. Immunocytochemical localization of beta-endorphin-containing neurons in the rat brain. *Neuroendocrinology* 1981 Jul;33(1):28–42. [PubMed: 6265819]
50. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, et al. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol* 2003 Jul;24(3):151–180. [PubMed: 14596810]
51. Koob GF, Le Moal M. Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* 2001 Feb;24(2):97–129. [PubMed: 11120394]
52. Brown SA, Vik PW, Patterson TL, Grant I, Schuckit MA. Stress, vulnerability and adult alcohol relapse. *J Stud Alcohol* 1995 Sep;56(5):538–545. [PubMed: 7475034]
53. Brady KT, Randall CL. Gender differences in substance use disorders. *Psychiatr Clin North Am* 1999 Jun;22(2):241–252. [PubMed: 10385931]

Figure 1. Ethanol consumption and preference of preproenkephalin and β -endorphin deficient and wild type mice

In all strains females consumed more ethanol than males. Alcohol consumption and preference of female END-KO animals was significantly lower than in the other two strains. The male END-KO mice consumed the lowest amount of EtOH compared to WT and ENK-KO males although END-KO and WT animals showed the same preference for alcohol. Thus, the combined liquid consumption was higher in WT compared to END-KO mice. The male ENK-KO mice showed significantly higher preference for alcohol in the first three weeks of the bottle choice session. ($n = 20/\text{gender/strain}$)

Figure 2. Stress-induced alcohol drinking

a) WT animals consumed significantly more ethanol and showed significantly higher preference for alcohol 24 hours after exposure to a mild foot shock. This effect was transient. Ninety-six hours later they consumed the same amount and showed the same preference as before the shock procedure (n=20). The ENK-KO mice consumed significantly lower alcohol 24 and 96 hours after the shock (n=20). **b)** The END-KO mice showed no changes in ethanol preference and consumption after the mild foot shock (n=20).

Figure 3. Location and linkage-disequilibrium of genotyped SNPs
The gene structure for the human PENK and POMC genes and the location of the analysed SNPs are indicated. The coding regions are plotted in orange. The transcription template for both genes is the negative strand. The linkage disequilibrium based on the German control group reveals one Haplotype block for PENK (marker 2 3 4: 19 kb) and two for POMC (marker 2 3: 2 kb; marker 4 5: 4 kb). LD-plots were generated using Haploview (www.broad.mit.edu/mpg/haploview/) with the Gold-heatmap colour scheme and r-square values were indicated.

Table 1
Development of tolerance to ethanol after chronic alcohol exposure
Intraperitoneal ethanol treatment caused dose-dependent hypothermia in alcohol naïve animals, which was significant at a dose 2 g/kg and 4 g/kg. WT and ENK-KO animals developed tolerance during chronic EtOH treatment, only 4 g/kg alcohol injection caused significant hypothermia (n=5).

	WT		ENK-KO		END-KO	
	Naïve	Chronic	Naïve	Chronic	Naïve	Chronic
Saline	0.13±0.24	0.55±0.38	0.54±0.24	0.42±0.35	0.11±0.27	0.32±0.29
1g/kg	-0.25±0.24	-0.25±0.23	0.35±0.24	0.02±0.35	-0.83±0.25	-0.87±0.31
2g/kg	-2.05±0.24 *	-0.68±0.35 #	-1.59±0.24 *	-0.84±0.35 #	-3.50±0.26 *	-3.00±0.22 *
4g/kg	-4.44±0.25 *	-4.57±0.45 *	-3.75±0.25 *	-3.02±0.35 *	-4.50±0.23 *	-5.01±0.28 *

* Significant difference compared to saline-treated group

significant difference compared to ethanol naïve group

Physical withdrawal symptoms after one month of forced drinking following three hours of withdrawal
The frequencies of higher HIC-scores were significantly increased three hours after withdrawal comparing to the EtOH naïve and EtOH drinking animals. Naïve: HIC-scores of EtOH naïve animals (n=10/strain), EtOH: HIC-scores during the drinking phase (WT: n=80, ENK-KO: n=40, END-KO: n=26), Withdrawal: three hours after replacing ethanol with water (WT: n=80, ENK-KO: n=40, END-KO: n=26).

		HIC-score Frequencies			
		0	1	2	3
WT	naïve	50	50	0	0
	EtOH	42.5	56.3	1.25	0
	withdrawal	1.25	17.5	45	36.25
ENK-KO	naïve	50	50	0	0
	EtOH	75	22.5	2.5	0
	withdrawal	25	37.5	35	0
END-KO	naïve	78	22	0	0
	EtOH	28	60	12	0
	withdrawal	8	44	44	4

Table 3
SNPs major allele frequency and association with alcohol dependence in the German sample

Gene	Marker	SNP ^a	genomic location ^b	Sample	Controls	Cases	p-value ^c	OR	C.I. ^d
PENK	rs12545109	C/A	57476460	all	0.773	0.712	0.031	0.729	[0.546–0.972]
		C/A		female	0.769	0.708	0.260	0.729	[0.420–1.265]
		C/A		male	0.774	0.712	0.060	0.723	[0.515–1.014]
PENK	rs239576581	G/A	57528504	all	0.515	0.568	0.094	1.242	[0.963–1.602]
		G/A		female	0.418	0.586	0.007	1.971	[1.206–3.222]
		G/A		male	0.552	0.565	0.729	1.054	[0.782–1.422]
PENK	rs12548084	C/T	57540810	all	0.946	0.945	0.924	0.973	[0.560–1.694]
		C/T		female	0.948	0.954	0.820	1.139	[0.372–3.485]
		C/T		male	0.946	0.941	0.800	0.921	[0.486–1.745]
PENK	rs2670029	C/A	57547550	all	0.569	0.606	0.239	1.165	[0.903–1.503]
		C/A		female	0.478	0.585	0.082	1.539	[0.946–2.504]
		C/A		male	0.603	0.615	0.759	1.048	[0.776–1.415]
POMC	rs2164808	G/A	25230680	all	0.557	0.496	0.056	1.276	[0.993–1.640]
		G/A		female	0.612	0.462	0.014	1.840	[1.128–3.002]
		G/A		male	0.536	0.506	0.413	1.130	[0.843–1.515]
POMC	rs7589318	G/A	25231876	all	0.678	0.651	0.368	0.885	[0.679–1.154]
		G/A		female	0.701	0.600	0.084	0.638	[0.383–1.063]
		G/A		male	0.669	0.668	0.956	0.992	[0.727–1.353]
POMC	rs1866146	T/C	25234077	all	0.670	0.669	1.000	0.997	[0.764–1.301]
		T/C		female	0.664	0.636	0.635	0.885	[0.534–1.465]
		T/C		male	0.672	0.683	0.767	1.049	[0.766–1.435]
POMC	rs6713532	T/C	25238337	all	0.771	0.798	0.308	1.171	[0.864–1.588]
		T/C		female	0.739	0.754	0.779	1.083	[0.622–1.886]
		T/C		male	0.783	0.816	0.280	1.224	[0.848–1.765]
POMC	rs934778 ^e	T/C	25242728	all	0.692	0.732	0.088	1.217	[0.971–1.525]
		T/C		female	0.693	0.786	0.004	1.629	[1.165–2.279]
		T/C		male	0.690	0.685	0.896	0.979	[0.713–1.344]
POMC	rs3769671 ^e	A/C	25243657	all	0.967	0.974	0.404	1.298	[0.701–2.403]
		A/C		female	0.974	0.970	0.725	3.969	[0.487–2.812]
		A/C		male	0.957	0.978	0.124	1.959	[0.820–4.681]

Gene	Marker	SNP ^a	genomic location ^b	Sample	Controls	Cases	p-value ^c	OR	C.I. ^d
POMC	rs6545976	G\T	25248154	all	0.967	0.959	0.489	0.79	[0.404–1.543]
		G\T		female	0.970	0.961	0.683	0.757	[0.199–2.884]
		G\T		male	0.966	0.958	0.566	0.798	[0.368–1.729]
POMC	rs6719226	C\G	25249516	all	0.966	0.959	0.571	0.827	[0.428–1.598]
		C\G		female	0.970	0.961	0.683	0.757	[0.199–2.884]
		C\G		male	0.964	0.958	0.666	0.847	[0.397–1.806]
POMC	rs874401	C\T	25255784	all	0.812	0.837	0.303	1.189	[0.855–1.652]
		C\T		female	0.791	0.800	0.856	1.057	[0.581–1.922]
		C\T		male	0.819	0.852	0.240	1.268	[0.853–1.885]

^aThe allele frequency is shown for the left allele.

^bGenomic location is based on NCBI human genome assembly B36.

^cUncorrected P-value of FAMHAP statistic for association between SNPs and alcohol dependence.

^d95% confidence interval

^eNote that the sample size is different for these markers

Table 4
SNPs genotype distribution and association with alcohol dependence in the German sample

Gene	Marker	Genomic location ^a	Sample	Controls	Cases	P (global) ^b	P (Armitage) ^c
PENK	rs12545109	57476460	all	CC=148 AC=81	CC=126 AC=97	0.104	0.035
			female	CC=39 AC=25	CC=33 AC=26	0.475	0.260
PENK	rs2576581	57528504	male	CC=109 AC=56	CC=92 AC=71	0.152	0.068
			all	GG=63 AG=120	GG=85 AG=104	0.103	0.104
PENK	rs12548084	57540810	female	GG=11 AG=34	GG=24 AG=27	0.019	0.009
			male	GG=52 AG=86	GG=61 AG=77	0.499	0.736
PENK			all	CC=218 CT=22	CC=218 CT=27	0.288	0.924
			female	CC=60 CT=7	CC=59 CT=6	0.815	0.815
PENK	rs2670029	57547550	male	CT=15 AC=124	CT=21 AC=111	0.228	0.805
			all	CC=78 AC=13	CC=93 AC=41	0.343	0.242
POMC	rs2164808	25230680	female	CC=13 AC=38	CC=22 AC=32	0.155	0.072
			male	CC=65 AC=86	CC=71 AC=78	0.696	0.764
POMC	rs7589318	25231876	all	GG=75 AG=125	GG=65 AG=113	0.090	0.060
			female	GG=24 AG=34	GG=15 AG=30	0.039	0.015
POMC			male	GG=51 AG=91	GG=49 AG=83	0.507	0.420
			all	GG=117 AG=101	GG=110 AG=99	0.612	0.388
POMC	rs1866146	25234077	female	GG=33 AG=28	GG=26 AG=26	0.178	0.099
			male	GG=84 AG=73	GG=83 AG=73	0.999	0.956
POMC	rs6713532	25238337	all	TT=117 CT=97	TT=113 CT=102	0.848	1.000
			female	TT=28 CT=33	TT=26 CT=32	0.832	0.621
POMC			male	TT=89 CT=64	TT=87 CT=69	0.701	0.783
			all	TT=151 CT=79	TT=158 CT=75	0.572	0.326
POMC	rs934778 ^d	25242728	female	TT=37 CT=25	TT=36 CT=26	0.778	0.777
			male	TT=114 CT=54	TT=122 CT=48	0.592	0.306
POMC	rs3769671 ^d	25243657	all	TT=195 CT=191	TT=178 CT=130	0.146	0.082
			female	TT=107 CT=120	TT=97 CT=45	0.0004	0.003
POMC			male	TT=88 AA=382	TT=80 CT=84	0.283	0.896
			all	CC=1 AC=25	CC=0 AC=17	0.561	0.409
POMC			female	CC=0 AC=12	CC=0 AC=9	0.722	0.722
			male	CC=1 AC=13	CC=0 AC=8	0.294	0.134

Gene	Marker	Genomic location ^d	Sample	Controls	Cases	P (global) ^b	P (Armitage) ^c
POMC	rs6545976	25248154	all	GG=228 GT=16	GG=223 GT=20	TT=0 TT=0	0.480
			female	GG=63 GT=4	GG=59 GT=5	TT=0 TT=0	0.677
			male	GG=165 GT=12	GG=163 GT=15	TT=0 TT=0	0.558
POMC	rs6719226	25249516	all	CC=231 CG=15	CC=222 CG=20	GG=0 GG=0	0.575
			female	CC=63 CG=4	CC=59 CG=5	GG=0 GG=0	0.677
			male	CC=168 CG=11	CC=162 CG=15	GG=0 GG=0	0.671
POMC	rs874401	25255784	all	CC=165 CT=71	CC=173 CT=64	TT=11 TT=8	0.316
			female	CC=41 CT=24	CC=43 CT=18	TT=2 TT=4	0.858
			male	CC=124 CT=47	CC=130 CT=45	TT=9 TT=4	0.255

^a Genomic location is based on NCBI human genome assembly B36.

^b Uncorrected P-value of FAMHAP statistic for association between SNPs and alcohol dependence.

^c P-value of the Armitage's trend test

^d Note that the sample size is different for these markers

Table 5
Haplotype frequencies and association of POMC with alcohol dependence in the German and Swedish sample

Sample	Marker		German		Swedish	
	rs934778	rs3769671	Controls	Cases	Controls	Cases
all	C	A	0.28	0.25	0.26	0.21
	T	A	0.69	0.73	0.70	0.74
females	C	C	0.03	0.02	0.04	0.04
	C	A	0.28	0.19	0.27	0.18
male	T	A	0.69	0.78	0.69	0.75
	C	C	0.02	0.02	0.03	0.07
	C	A	0.27	0.29	0.27	0.24
	T	A	0.69	0.68	0.69	0.74
	C	C	0.04	0.02	0.04	0.02
						0.12
						0.077
						0.012
						0.016