The 1258 G>A polymorphism in the neuropeptide Y gene is associated with greater alcohol consumption in a Mediterranean population

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Abstract

Neuropeptide Y (NPY) is a neurotransmitter widely distributed in the central nervous system. Several studies have demonstrated that increases of NPY are associated with reduced alcohol intake and anxiety manifestations. The Leu7Pro polymorphism in the NPY has been associated with alcohol consumption, but evidence is scarce. In the Spanish Mediterranean population, this variant is not polymorphic. Thus, our aim is to identify novel functional variants in the NPY and to investigate the impact of these markers and others previously described on alcohol consumption in this population. A total of 911 subjects (321 men and 590 women) from the Spanish Mediterranean population were recruited. Alcohol consumption, and demographic and lifestyle variables were measured. Nucleotide sequence determination and SNP analyses were carried out. Only one exonic SNP was detected by direct sequencing (1258 G>A or rs9785023; allele frequency 0.47). From the intronic markers chosen (483 A>G or rs13235938, 2517 A>G or rs4722342, and 7065 A>G or rs4722343), only the two latter ones were polymorphic (allele frequencies 0.46 and 0.04, respectively), and none of them were associated with alcohol consumption. However, the 1258 G>A SNP was associated (recessive pattern) with higher alcohol intake. This association was particularly relevant in men with high alcohol intake (59.1 ± 5.0 g/day in AA as opposed to 40.6 ± 7.5 in the G carriers, \(P = .022\)) and women with moderate alcohol intake (7.3 ± 5.5 g/day in AA as opposed to 4.6 ± 3.9 g/day in G carriers, \(P = .048\)). The 1258 G>A polymorphism in the NPY is associated with higher alcohol consumption in the Mediterranean population. © 2011 Elsevier Inc. All rights reserved.

Keywords: Alcohol consumption; Neuropeptide Y; Genetic polymorphism

Introduction

Overconsumption of alcohol is a drug abuse commonly associated with anxiety and depression (Kushner et al., 1999). Animal models have shown that acute ethanol administration produces dose-dependent anxiolytic effects (Wilson et al., 2004). In the same models, greater alcohol intake has been associated with states of anxiety (Spanagel et al., 1995).

Neuropeptide Y (NPY) is a neurotransmitter widely distributed in the central nervous system (Gray and Morley, 1986). This 36-amino acid polypeptide is a neuro-modulator that has been linked to feeding and anxiety-associated behaviors (Baraban, 1998). NPY seems to have central anxiolytic functions (Primeaux et al., 2005). The hippocampal NPY system is downregulated during ethanol withdrawal anxiety states (Olling et al., 2007). Both intra-amygdalar injection and overexpression experiments in animals have demonstrated that increases of NPY in the amygdala reduces alcohol intake and anxiety manifestations in rats (Primeaux et al., 2006; Thorsell et al., 2007).

In humans, the implication of central NPY levels in alcohol consumption is little known, but the brain tissue from alcoholics has significantly lower NPY expression than brain tissue from controls (Mayfield et al., 2002). However, it is not clear if the decreased NPY levels observed were present before alcoholism began or are the consequence of alcohol consumption.

Most studies on humans, however, have investigated the effect of NPY genetic variations on alcohol consumption. These studies have mainly been focused on the Leu7Pro (1128T>C) polymorphism located in the signal peptide of the prepro-NPY (Karvonen et al., 1998). The effect of this polymorphism on plasmatic NPY levels is little known and evidence is contradictory. Thus, Kallio et al. (2001) found that individuals with the Leu7/Pro7 genotype had an average of 42% higher maximal increases of plasma NPY in response to physiological stress compared with...
Leu7/Leu7 individuals. More recent studies have shown that carriers of the 7Pro allele have lower plasma NPY levels (Kallio et al., 2003). Yet, another study suggests that this polymorphism does not affect plasmatic NPY levels (Jaakkola et al., 2007).

Association studies involving the effects of these polymorphisms on the risk of alcoholism are also confusing. Although, some studies associate the presence of the 7Pro variant with an increased alcoholism risk (Lappalainen et al., 2002) and increasing ethanol consumption (Kauhanen et al., 2000), others found a lower frequency of the 7Pro allele in alcoholics (Ilveskoski et al., 2001). Furthermore, Zhu et al. (2003) and Zill et al. (2008) did not find significant differences of genotype frequencies between alcoholics and nonalcoholics from Finland and Sweden, and Germany, respectively.

In the Spanish Mediterranean population, this variant is not polymorphic as we reported in a previous study (Portolés et al., 2003). For this reason, it is necessary to assess the prevalence of other polymorphisms in this gene, by studying those previously described in the literature and undertaking a screening of the exonic regions of the NPY gene in this population.

Thus, our aims are (1) to undertake a genetic screening of the four exons of the NPY gene to find new polymorphic variants in this population. (2) To estimate the prevalence of other functional or intronic variants in the NPY in the Mediterranean population. (3) To estimate the association between these genetic variants and alcohol consumption.

Material and methods

Subjects

In the present study, 911 unrelated Caucasian subjects (321 men and 590 women) from the Mediterranean population were analyzed. These subjects were recruited in a previous study aimed at investigating the influence of various genetic polymorphisms on obesity, using a case—control design (Portolés et al., 2006). The sample was composed of 608 controls (nonobese) from general population and 303 obese individuals (body mass index ≥30 kg/m²) paired by age and gender. Half of the obese individuals were also recruited from the general population in a primary health care center, and the other half were recruited at the endocrinology unit of a hospital in the same area. All subjects (aged 18—85 years) were randomly recruited from the Valencia Region on the East Mediterranean coast of Spain. All of them provided informed consent. The study protocol was approved by the Ethics Committee of the School of Medicine of the University of Valencia, Spain.

DNA extraction and genotyping

From each participant, a peripheral venous blood sample was obtained and genomic DNA was isolated by standard methods.

A PCR—RFLP protocol was developed to genotype the entire sample for the genetic markers found in the exon screening and three extra intronic genetic markers found in genetic databases. PCR was carried out in an Eppendorf DNA thermal cycler. Table 1 shows primers, and restriction enzymes used in the genetic markers genotyping.

Sequencing of PCR products

We carried out a direct sequencing screening in a 50-individual subsample by using a genetic analyzer (ABI Prism TM 310; PE Applied Biosystems). Table 1 shows the primers used in the exon sequencing. This sample included the top 40 individuals with greater alcohol consumption of the sample and a random sample of 10 individuals who did not
consume alcohol, to increase the probability of detecting genetic variants associated with the extreme phenotypes.

### Alcohol consumption and demographic, clinical, and lifestyle variables

A number of sociodemographic and behavioral variables such as gender, age, educational level, marital status, and tobacco smoking were assessed to appraise their potential confounding effect on the association study between the polymorphisms and alcohol consumption.

Alcohol consumption was carefully evaluated by a set of 22 questions about the use of alcoholic beverages during workdays and weekends, as previously described (Corella et al., 2000). In that questionnaire, week and weekend consumption of a list of alcoholic beverages including beer, white wine, red wine, champagne, brandy, whisky, vodka, anisette, and martini were assessed. The mean daily ethanol consumption (in grams) was calculated by multiplying the amount consumed (in milliliters) by the percentage of ethanol supplied by each specific beverage according to the alcoholic graduation equivalence table (Cuevas-Badenes and Sanchis-Fortea, 2000). From the reported alcoholic beverages, alcohol consumption was considered as a continuous variable expressed in grams per day. In addition, alcohol consumption was categorized as a drinker variable: nonalcohol consumers (no alcohol consumption) and alcohol consumers (any amount of alcohol consumed). Finally, among alcohol consumers, three categories of alcohol consumption were considered (Cuevas-Badenes and Sanchis-Fortea, 2000): moderate consumption (<30 g alcohol/day for men and <20 g alcohol/day for women); high consumption (>30/<75 g alcohol/day for men and >20/<60 g alcohol/day for women); and very high consumption alcoholism (>75 g alcohol/day for men and >60 g/day for women).

### Statistical analysis

Hardy-Weinberg equilibrium was assessed by using the Chi-square test. To assess the associations with alcohol consumption, codominant, dominant, and recessive genetic models were fitted. To estimate associations with the quantitative alcohol intake (grams per day), analysis of variance, t Student, and logistic regression models were performed. For these tests, a logarithmic transformation of alcohol intake was carried out. The alcohol consumption risk was estimated by logistic regression models calculating the odds ratio (OR) and its confidence interval (CI). Crude and covariate-adjusted models (adjusted for age, civil status, educational level, and tobacco smoking) were carried out. Statistical analyses were performed using SPSS software package (SPSS Inc, Chicago, IL). Significance level of bilateral P was considered statistically significant under P < .05.

### Results

General characteristics of the studied sample depending on the alcohol consumption and gender are depicted in Table 2. Mean daily alcohol consumption was higher in men (14.2 ± 16.5 g/day) than in women (3.4 ± 6.3 g/day).

#### Table 2

Means ± S.D. and proportions of clinical and sociodemographic variables in the studied sample depending on alcohol intake

<table>
<thead>
<tr>
<th>Alcohol consumers (n = 562)</th>
<th>Nonalcohol consumers (n = 349)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men (264)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46.6 ± 12.3</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>28.3 ± 5.3</td>
</tr>
<tr>
<td>Obesity (BMI ≥ 30)</td>
<td>87 (32.9%)</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>133 (50.6%)</td>
</tr>
<tr>
<td>Education level, n (%)</td>
<td></td>
</tr>
<tr>
<td>Illiterate</td>
<td>5 (1.7%)</td>
</tr>
<tr>
<td>Primary</td>
<td>131 (50.7%)</td>
</tr>
<tr>
<td>Secondary</td>
<td>158 (58.8%)</td>
</tr>
<tr>
<td>University</td>
<td>76 (27.6%)</td>
</tr>
<tr>
<td>Marital status, n (%)</td>
<td></td>
</tr>
<tr>
<td>Unmarried</td>
<td>46 (15.8%)</td>
</tr>
<tr>
<td>Married</td>
<td>216 (73.9%)</td>
</tr>
<tr>
<td>Divorced</td>
<td>6 (2.4%)</td>
</tr>
<tr>
<td>Widow</td>
<td>24 (8.2%)</td>
</tr>
<tr>
<td>Alcohol consumption (g/day)</td>
<td></td>
</tr>
<tr>
<td>Moderate consumption (n = 341)</td>
<td>—</td>
</tr>
<tr>
<td>High consumption (n = 216)</td>
<td>—</td>
</tr>
<tr>
<td>Alcoholic consumption (n = 5)</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup>P values obtained in the comparison between men and women in each alcohol consumption group. Moderate alcohol consumption (<30 g alcohol/day for men and <20 g alcohol/day for women); high consumption (>30/<75 g alcohol/day for men and >20/<60 g alcohol/day for women); and alcoholic consumption (>75 g alcohol/day for men and >60 g/day for women).
Among alcohol consumers, most of the women had moderate alcohol consumption. We detected a very low prevalence of excessive alcohol consumption in our sample (only 5 men and 0 women). However, this is to be expected given that we are dealing with a general population sample.

As a result of the direct sequencing of the four NPY exons, only one exonic polymorphism was detected. This genetic marker was the 1258 G > A (rs9785023 or Ser50Ser), allele frequency 0.47), located in exon 2, and had been previously described (Okubo and Harada, 2001). Table 3 shows the distribution of the 1258 G > A alleles in alcohol consumers and nonalcohol consumers. Genotype distribution was in accordance with Hardy–Weinberg equilibrium ($P = .11$). Furthermore, following a genomic database research, we chose three intronic variants to test their variability in our sample. Of the three intronic markers chosen (rs13235938 or 483 A > G, rs4722342 or 2517 A > G and rs4722343 or 7065 A > G), two of them (rs4722342 and rs4722343) were shown to have variability in our sample (allelic frequencies were 0.46 and 0.04, respectively, for the variant alleles). Genotype distribution of both intronic polymorphisms was in accordance with Hardy–Weinberg equilibrium ($P = .98$ and $P = .15$, respectively).

No association was found between the distribution of the three polymorphisms and the alcoholic habit (Table 3). However, when alcohol consumption was dichotomized according to the mean of the population as cut-off point, and after covariate adjustment, carriers of the AA genotype of the 1258 G > A polymorphism were found to be more frequent in the over-the-mean alcohol intake group in men (OR = 2.85; 95% CI: 1.03–7.84; $P = .043$). Further, we studied the association of these markers with the daily pure alcohol consumption.

In both men and women, AA homozygotes had a higher daily alcohol intake than carriers of the G allele, but these values did not reach statistical significance. When alcohol consumption groups (moderate intake, high intake, and pure alcohol consumption) were studied separately, and after covariate adjustment (age, tobacco smoking, educational level, and civil status), we found statistically significant associations. Thus, for men in the high consumption group (>30/75 g alcohol/day), we found a statistically significant association between the 1258 G > A polymorphism and the amount of alcohol consumed: 40.6 ± 7.5 g/day in carriers of the G allele versus 59.1 ± 5.0 g/day in AA homozygotes; $P = .022$ (Fig. 1). In women, we also found a statistically significant association between the 1258 G > A polymorphism and the amount of alcohol consumed. This association was detected in the group of moderate alcohol consumption (<20 g/day): 4.6 ± 3.9 g/day in carriers of the G allele versus 7.3 ± 5.5 g/day in AA homozygotes; $P = .048$ (Fig. 2). When we analyzed the two other intronic polymorphisms, no significant associations were detected, either in the crude analysis or in the stratified approach.

**Discussion**

We have found an increased alcohol consumption in subjects homozygous for the A allele of the 1258 G > A polymorphism in the NPY gene, in subjects from the

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**Table 3**

Distribution of 1258 G > A genotypes in alcohol consumers and nonalcohol consumers

<table>
<thead>
<tr>
<th>NPY genotypes</th>
<th>Nonalcohol consumers</th>
<th>Alcohol consumers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1258 G &gt; A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G, n (%)</td>
<td>107 (30.7%)</td>
<td>137 (24.4%)</td>
</tr>
<tr>
<td>G/A, n (%)</td>
<td>168 (48.1%)</td>
<td>310 (55.1%)</td>
</tr>
<tr>
<td>A/A, n (%)</td>
<td>74 (21.2%)</td>
<td>115 (20.5%)</td>
</tr>
<tr>
<td>2517 A &gt; G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G, n (%)</td>
<td>108 (30.9)</td>
<td>157 (27.9)</td>
</tr>
<tr>
<td>G/A, n (%)</td>
<td>172 (49.3)</td>
<td>281 (50.0)</td>
</tr>
<tr>
<td>A/A, n (%)</td>
<td>69 (19.8)</td>
<td>124 (22.1)</td>
</tr>
<tr>
<td>7065 A &gt; G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A, n (%)</td>
<td>321 (91.8)</td>
<td>507 (90.2)</td>
</tr>
<tr>
<td>G/A, n (%)</td>
<td>28 (8.2)</td>
<td>55 (9.8)</td>
</tr>
<tr>
<td>G/G, n (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

*P value obtained by the $\chi^2$ test in comparing genotype frequencies between alcohol consumers and nonalcohol consumers.
Spanish Mediterranean population. This association was consistent and in the same direction in both men and women. In women, this association was statistically significant in the moderate alcohol consumption group (the most prevalent). In men, this association reached the statistical significance in the high alcohol consumption group. In both, the presence of the AA genotype represented an increase in alcohol consumption that surpassed G-allele carrier consumption by over 30%. Although recruitment was initially made for another phenotype, most of the sample was made up from individuals randomly selected from the general population, then providing a valid sample for the identification study of new genetic variants in the NPY gene, and for the association study of the NPY variants with alcohol consumption.

As has been said before, studies assessing the association between genetic variations in the NPY gene have focused on the 1128 T>C polymorphism, and the results are contradictory. The absence of the 1128 T>C polymorphism in our Spanish Mediterranean population has been previously reported (Portolés et al., 2003). Our exon screening revealed the existence of one frequent variant in that population (1258 G>A), but did not detect any more. Therefore, we decided to genotype three previously described intronic variants, of which only two were polymorphic. In the association analysis of these two variants, together with the exonic one, with the fact of consuming alcohol or not, we did not find any statistically significant association. This observation strengthens the hypothesis that the fact of consuming alcohol or not, is less likely to be under genetic control than the quantity of alcohol consumed. With regard to the associations of these variants with the quantity of alcohol consumed, we have indeed found an important association of the exonic variant (1258 G>A). The association we have found between this marker and alcohol consumption shows some differences in the amount of alcohol consumed by gender. The association becomes evident in women in the moderate consumption group and men in the high consumption group. A possible reason for that phenomenon is the social component that alcohol consumption retains in the case of men and is not present, or is much lower, in women. This constant, nongenetically determined amount of social alcohol intake may be the cause of this displacement to the next consumption group observed in men. The fact that there were no associations in the maximum (alcoholic) consumption category may suggest that other psychological, environmental, or genetic factors may be involved. However, the very low prevalence of alcohol consumption in the alcoholic range in our general population sample (only 5 men and 0 women), may be the main limitation due to the lack of statistical power.

The mechanism underlying the predisposing effect of the A allele of the 1258 G>A polymorphism in a recessive model to a higher alcohol consumption is unknown, as is the case of the Leu7Pro polymorphism. Influence of the latter genetic variation on plasmatic NPY levels is uncertain (Jaakkola et al., 2007; Kallio et al., 2001, 2003). These genetic variations may not affect NPY expression, but can alter the affinity or the neurotransmitter–receptor spatial relationship, leading to a reduction in the active NPY availability in the amygdala, in line with the observed phenomena in animal models.

The 1258 G>A polymorphism has been little studied previously (Bhaskar et al., 2007; Okubo and Harada, 2001), and no associations among this mutation and alcoholism risk or alcohol consumption were found. The first study was undertaken on a Japanese population, and the potentially important differences in exposure to other genetic and environmental factors may justify the divergences between our results and those obtained from this sample. The second work only investigated the prevalence of NPY variants in 14 Indian samples. Thus, our results enable us to conclude that the 1258 G>A polymorphism may be an important genetic marker of high alcohol consumption level in Mediterranean populations. However, for generalization, these results require replication in other independent populations. Due to its synonymous coding, this marker must be in linkage disequilibrium with another functional variant. In line with this, 1258 G>A polymorphism has been demonstrated to be in linkage disequilibrium with 1128 T>C (Bhaskar et al., 2007) and 5671 C>T (Okubo and Harada, 2001). These polymorphisms seem to be little prevalent in our population and have not been detected in our exon screening. Finally, with regard to the intronic markers analyzed, this is the first work that studies the impact of these markers on alcohol consumption. Our results suggest that these polymorphisms have no influence on this habit.

New studies extending screening to the promoter and the entire intronic regions will enable us to better understand the impact of genetic variations in the NPY gene on alcohol consumption.

Acknowledgments

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References


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