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Genetic variation and epigenetic modification of the prodynorphin gene in peripheral blood cells in alcoholism

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ABSTRACT

Dynorphins are critically involved in the development, maintenance and relapse of alcoholism. Alcohol-induced changes in the prodynorphin gene expression may be influenced by both gene polymorphisms and epigenetic modifications. The present study of human alcoholics aims to evaluate DNA methylation patterns in the prodynorphin gene (*PDYN*) promoter and to identify single nucleotide polymorphisms (SNPs) associated with alcohol dependence and with altered DNA methylation. Genomic DNA was isolated from peripheral blood cells of alcoholics and healthy controls, and DNA methylation was studied in the *PDYN* promoter by bisulfite pyrosequencing. In alcoholics, DNA methylation increased in three of the seven CpG sites investigated, as well as in the average of the seven CpG sites. Data stratification showed lower increase in DNA methylation levels in individuals reporting craving and with higher levels of alcohol consumption. Association with alcoholism was observed for rs2235751 and the presence of the minor allele G was associated with reduced DNA methylation at *PDYN* promoter in females and younger subjects. Genetic and epigenetic factors within *PDYN* are related to risk for alcoholism, providing further evidence of its involvement on ethanol effects. These results might be of relevance for developing new biomarkers to predict disease trajectories and therapeutic outcome.

1. Introduction

Alcohol abuse causes about 1.8 million deaths a year (3.2% of all deaths worldwide), and alcohol dependence with associated health problems is the third most common cause of death in developed countries (Rehm et al., 2004). Continued excessive alcohol consumption can lead to the development of alcohol dependence, characterized by physiological withdrawal when alcohol consumption is substantially reduced. Unpleasant withdrawal effects contribute to relapse, estimated to occur in 50–90% of abstinence attempts, and are clearly a major impediment to treatment efforts (Loosen et al., 1990; Powell et al., 1998; Finney et al., 1999; Ciraulo et al., 2003; Moos & Moos, 2006; Charney et al., 2010). Increasing evidence supports a link between alcohol consumption and the endogenous opioid system, which is proven to be important when considering positive reinforcing effects following acute alcohol consumption and the downstream neuroadaptations following chronic alcohol exposure (Walker et al., 2012). In animal models, acute alcohol stimulates endogenous opioid peptide release (Lam et al., 2008), whereas a central opioid deficiency has been observed during heavy alcohol consumption, which might further promote alcohol intake through negative reinforcement. β-endorphin and enkephalin, endogenous ligands of the mu-opioid receptor (MOP) and delta-opioid receptor (DOP), respectively, have been associated with the euphoric/rewarding effects of alcohol consumption (Nealey et al., 2011).

In contrast, dynorphins (DYNs), endogenous ligands of the kappa-opioid receptor (KOP) (Chavkin et al., 1982) induce dysphoric/anhedonic effects in humans and aversive behaviors in animals (Nealey et al., 2011), contributing to excessive alcohol seeking, similar to that observed in alcohol dependence (Walker and Koob, 2008). It has also been suggested that the DYNs/KOP system may be a key mediator in the stress-related effects of alcohol (Walker et al., 2012). Alcohol-induced changes in the prodynorphin gene (*PDYN*) expression have been linked to neuroplastic adaptations critical for addiction (Bazov et al., 2013; Butelman et al., 2012; D'Addario et al., 2008 and D'Addario et al., 2013; Shippenberg et al., 2007; Taqi et al., 2011a; Walker and

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Koob, 2008; Wee and Koob, 2010), contributing to the impairment of cognitive control over alcohol drinking behavior (Shippenberg et al., 2007; Taqi et al., 2011a and 2011b). Changes in the *PDYN* gene expression may be influenced by gene polymorphisms and epigenetic mechanisms. It is, in fact, known that development of alcoholism is influenced by both genetic and environmental factors (Gelernter and Kranzler, 2009; Kimura and Higuchi, 2011), and that gene-environment interactions can be mediated by epigenetic regulation of gene expression. The *PDYN* gene remains a strong candidate for alcohol dependence-related phenotypes in humans, even though genetic studies on the link between *PDYN* variation and alcohol dependence have demonstrated both association (Karpyak et al., 2013; Williams et al., 2007; Xuei et al., 2006) as well as lack of association (Bierut et al., 2010; Edenberg et al., 2010; Treutlein et al., 2009). However, the effects of previously detected genetic risk factors for psychiatric disorders, including alcohol dependence, remain small.

We explore here the hypothesis that the influence of alcohol consumption on human physiology might be due to a combination of genetic and environmental factors, which are mediated by epigenetic mechanisms. DNA methylation is one of the most studied epigenetic modifications, associated with reduced gene expression, and is known to be involved in numerous biological processes (Bird, 2002). Genetic variations have been shown to influence the inter-individual variation in DNA methylation (Zhang et al., 2010). However, the impact of genetic variations on the DNA methylation pattern of most genes is not fully understood. The aim of our study is to identify the genetic and epigenetic regulation of *PDYN* in alcoholism by: (A) analysis of DNA methylation of the proximal promoter region of *PDYN* in peripheral blood cells from alcoholics and healthy controls; and (B) detection of single nucleotide polymorphisms (SNPs) in the *PDYN* gene associated with alcohol dependence and/or DNA methylation changes. The availability of a well-defined cohort of alcoholics allows us to use data stratification based on different characteristics of the study sample and thus to possibly assess disease development and trajectories.

2. Methods

2.1. Study subjects and data collection

This study was approved by the Regional Ethics Review Board in Stockholm, Sweden, for the alcoholic and control samples (Number: Dnr:01-392). All alcoholic subjects provided informed consent and permission to use their information for future studies of alcohol dependence and related phenotypes. Samples from 744 affected individuals (521 males of mean age 49.4 ± 10.7 years; 223 females of mean age 48.6 ± 10.4 years) and 1079 control subjects (301 males and 778 females of mean age 52 ± 12 years) of Scandinavian ancestry were included in the association study. Alcoholic patients were enrolled within the Swedish 'Alcoholism in Siblings' study. Alcohol dependence was defined according to DSM-IV criteria after a structural psychiatric interview conducted in accordance with the Schedules for Clinical Assessment in Neuropsychiatry (Wing et al., 1990). Subjects who additionally met DSM-IV criteria for dependence on an illicit drug or had recently (within one year) suffered any major psychiatric disorder were excluded. A history of psychiatric disorders, such as a depressive episode, did not lead to exclusion. The group of healthy controls consisted of individuals enrolled in the 'Epidemiological Investigation of Rheumatoid Arthritis (EIRA)' study (Plenge et al., 2007). These control individuals for the genetic association study were Swedish Caucasians from the general population between the ages of 18 and 70 years and were included in the study during the period 1996–2004. Data for alcohol consumption was available for 955 individuals (88.5%); 113 (11.8%) declared that they never consumed alcohol; for the remaining individuals, alcohol consumption was skewed to low values with a median of 48 g per week, IQR 50. Demographic and clinical characteristics of the alcoholic patient cohort are shown in Table 1.

Table 1
Demographic and Clinical Characteristics of the Study Sample.

Characteristic	N (%) or Mean ± SD		
	All Subjects	Males	Females
Race			
European Swedish/Norwegian/ Danish	744 (100%)	521 (70%)	223 (30%)
Age (years)	49.2 ± 10.6	49.4 ± 10.7	48.6 ± 10.4
Drinks/day (baseline)[a]	12.6 ± 5.8	12.8 ± 6.0	12.1 ± 5.5
Drinking days/month (baseline)	8.9 ± 8.4	8.8 ± 8.3	9.1 ± 8.6
Craving	252 (33.9%)	173 (23.2%)	79 (10.6%)
Withdrawal symptoms	392(52.7%)	281 (37.8%)	111 (14.9%)
Drink to relieve withdrawal	263 (67.8)	188 (48.4)	75 (19.3)
Delirium and convulsion	49 (12.6)	36 (9.3)	13 (3.4)
Tolerance	202 (52%)	149 (38.4%)	53 (13.7%)
Reversed tolerance	146 (37.6)	116 (29.9)	30(7.7)
Alcohol–induced psychosis	39 (5.2%)	26 (3.5%)	13 (1.7%)
Antidepressant users	34 (8.8%)	19 (4.9%)	15 (3.9%)
Antianxiety users	92 (23.7%)	48 (12.4%)	44 (11.3%)
Nicotine dependence	354 (47.5%)	201 (27.0%)	153 (20.6%)
Other substance dependence	159 (36.9%)	110 (39.7%)	49 (31.8%)
Cannabis	133 (17.9)	99 (13.3)	34 (4.6)
Cocaine	29 (3.9)	19 (2.6)	10 (1.4)
Hallucinogen	23 (3.1)	17 (2.3)	6 (0.8)
Sedatives	176 (23.7)	101 (13.6)	75 (10.1)
Stimulant	144 (19.4)	103 (13.8)	41 (5.5)
Opiate	31 (4.2)	21 (2.8)	10 (1.3)
Solvent abuse (sniffing)	54 (7.2)	44 (5.9)	10 (1.3)
Others	160 (2.7)	140 (18.8)	20 (2.7)

[a] Average number of drinks per drinking day.

2.2. SNP selection and genotyping

Genomic DNA was isolated as previously described (Geijer et al., 1994). Locations of the selected *PDYN* SNPs examined are shown in Fig. 1a. Candidate SNPs for genotyping were chosen to replicate previously reported associations with alcohol, opiate and/or cocaine dependence (Xuei et al., 2006; Yuferov et al., 2009). Non-alcoholic controls were genotyped as part of EIRA study (Plenge et al., 2007). TaqMan Genotyping Assays were applied for genotyping of SNPs (Life Technologies, Carlsbad, CA) for cases and controls. The end point fluorescence readings were performed using an ABI Prism 7900 System.

2.3. Analysis of DNA methylation by bisulfite pyrosequencing

A subset of 151 alcoholics and 88 healthy individuals was selected for the methylation studies to include individuals for statistical analysis that were representative of the total study population with regard to sex, age distribution, genotype and, for alcoholics, the phenotypes distribution. Methylation status of the *PDYN* promoter region was determined using pyrosequencing of bisulfite-converted genomic DNA isolated from blood cells. After DNA extraction, 0.5 μg of DNA from each sample was treated with bisulfite, using a DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was amplified by the PyroMark PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. *PDYN* primer sequences (Forward: GGAAGAGAGTAGGAAGATGATAG; biotin-Reverse: TTCTACCAAAACT AATATACCTAACACC; Sequencing: AGAGAGTAGGAAGATGATAGT) were provided by Qiagen. The sequence was from the forward strand and the assay was designed to target a region within the CpG island located upstream of the *PDYN* exon 1 (Fig. 1b) previously identified by Yuferov et al., 2011. PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 10 min. PCR products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen), which calculates