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Variants of opioid system genes are associated with non-dependent opioid use and heroin dependence

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Abstract

Background: Heroin addiction is a chronic, relapsing brain disease. Genetic factors are involved in the development of drug addiction. The aim of this study was to determine whether specific variants in genes of the opioid system are associated with non-dependent opioid use and heroin dependence.

Methods: Genetic information from four subject groups was collected: non-dependent opioid users (*NOD*) [n=163]; opioid-dependent (*OD*) patients in methadone maintenance treatment (*MMT*) [n=143]; opioid-dependent *MMT*-resistant patients in heroin-assisted treatment (*HAT*) [n=138]; and healthy controls with no history of opioid use (*HC*) [n=153]. Eighty-two variants in eight opioid system genes were studied. To establish the role of these genes in (a) non-dependent opioid use, and (b) heroin dependence, the following groups were compared: *HC* vs. *NOD*; *HC* vs. *OD* (*MMT*+*HAT*); and *NOD* vs. *OD* (*MMT*+*HAT*).

Results: Five unique SNPs in four genes showed nominally significant associations with non-dependent opioid use and heroin dependence. The association of the delta opioid receptor (*OPRD1*) intronic SNP rs2236861 with non-dependent opioid use (*HC* vs. *NOD*) remained significant after correction for multiple testing (OR=0.032; $p_{corrected} = 0.015$). This SNP exhibited

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Conflict of interest

The authors declare no conflict of interest with regard to the content of this paper.

a significant gene-gene interaction with prepronociceptin *(PNOC)* SNP rs2722897 (OR=5.24; $p_{corrected}$ = 0.041) (*HC* vs. *NOD)*.

Conclusions: This study identifies several new and some previously reported associations of variants with heroin dependence and with non-dependent opioid use, an important and difficult to obtain group not extensively studied previously. Further studies are warranted to confirm and elucidate the potential roles of these variants in the vulnerability to illicit drug use and drug addiction.

Keywords

heroin addiction; non-dependent opioid use; heroin-assisted treatment; opioid genes; rs2236861

1. Introduction

Heroin addiction is a chronic, relapsing brain disease that causes major medical, social and economic problems worldwide. Once exposed, there are at least three main factors that contribute to the development of heroin dependence: environmental, drug-induced and genetic factors. Various estimates place the genetic contribution to developing a drug addiction at 40–60% (Kendler et al., 2003; Tsuang et al., 1996), suggesting the possibility of multiple genetic variants being involved. Identification of the variants involved is important for the understanding of the causal pathways to addiction and for the improvement of its diagnosis and treatment. However, it has proved difficult, as with any phenotype complex as addiction, to determine the specific genes responsible. When conducting an association study, having control groups that are not well characterized is one possible cause for this difficulty. Genes associated with a liability for drug dependence but are not involved in the initial drug use would not necessarily display any effect in the absence of drug exposure (Nelson et al., 2013). Therefore, access to a group of exposed but non-dependent heroin (or other opioids) users is very useful in elucidating the stage of drug use that a particular gene is influencing. This study tries to address this issue by including a group of non-dependent opioid users in addition to the group of dependent opioid users and the group of drug-free subjects. Few studies comparing dependent subjects to subjects exposed but non-dependent have been reported (Nelson et al., 2012; Nelson et al., 2013).

The endogenous opioid system is a group of genes coding for the four major subtypes of 7-transmembrane, G protein-coupled opioid receptors and their opioid ligands. The subtypes of receptors are; mu, kappa, delta and receptor-like. The ligands of these receptors are the endogenous opioid peptides, which are encoded for by the genes; proopiomelanocortin (*POMC*), prodynorphin (*PDYN*), proenkephalin (*PENK*) and prepronociceptin (*PNOC*). This network of eight genes plays a key role in drug addiction and as such its gene variants are obvious candidates for a hypothesis-driven study of opioid addiction. Previous studies have found associations of multiple variants in opioid system genes with heroin addiction (Kreek et al., 2005a; Kreek et al., 2005b; Levran et al., 2008; Levran et al., 2012; Nelson et al., 2012; Zhang et al., 2008). Here, we report the results of an association study of 82 SNPs in the eight major opioid genes with non-dependent heroin (or other opioids) use and heroin dependence in a sample of Caucasian subjects from the Netherlands.

2. Materials and Methods

2.1. Subjects

Three groups were recruited in the Netherlands, comprising a total of 795 subjects (Blanken et al., 2009; Blanken et al., 2010; Korf et al., 2010; van den Brink et al., 2003; Zaaijer et al., 2014). as follows (Table 1):

- Group 1: Non-dependent opioid users (*NOD*) who reported a lifetime history of illicit use of opioids. Criteria included that volunteers had to be at least 25 years of age, used heroin (or other non-prescribed opioids) at least 5 times, but less than 100 times, with the first use at least 2 years ago, and never had been in treatment to reduce or stop their use of heroin (or other non-prescribed opioids). Recruitment was made through “convenience” sampling (i.e., advertisements in local media), as well as through “snowball” or “chain referral” (Korf et al., 2010; Zaaijer et al., 2014).
- Group 2: Opioid dependent heroin users (*OD*) meeting DSM-IV criteria for opioid dependence for at least five years were;
  - Heroin dependent subjects currently in methadone maintenance treatment (*MMT*).
  - *MMT*-resistant heroin-dependent subjects, currently in heroin-assisted treatment (*HAT*). Subjects in this group were co-prescribed injectable or inhalable pharmaceutical grade diacetylmorphine (heroin) plus oral methadone.
- Group 3: Healthy controls without a history of any illicit opioid use and no history of alcohol or drug dependence by DSM-IV criteria (*HC*). Nicotine dependence was not an exclusion criterion. Recruitment was through “convenience” sampling (i.e., advertisements in local media), as well as through personal contact, or referral by others.

2.2. Socio-demographic and drug use assessment

All subjects were extensively interviewed by a skilled clinical investigator in the Netherlands. Age, gender, and country of origin information were collected using a standard questionnaire. Subjects were administered the Kreek-McHugh-Schluger-Kellogg scale (KMSK)(Kellogg et al., 2003), a relatively rapid method to quantity self-exposure to opioids, cocaine, alcohol and tobacco. The KMSK scale assesses the frequency, amount, and duration of exposure to each substance during a person’s period of greatest use (lifetime score). In prior studies, the results of KMSK assessments were evaluated using receiver operator characteristics (ROC) analysis for the optimal cut-point score for alcohol, cocaine and opiate dependence/addiction diagnoses(Kellogg et al., 2003).

The Central Committee on Research Involving Human Subjects in the Netherlands (CCMO) approved the study of heroin-assisted versus methadone maintenance treatments and the human molecular genetics study for all study groups. The genetics study was also approved

by The Rockefeller University's Institutional Review Board (2004). All subjects signed informed consent for the genetics research.

2.3. Genotyping

Blood specimens were collected in the Netherlands and shipped to the Laboratory of the Biology of Addictive Diseases at The Rockefeller University, where DNA was extracted and quantified using standard methods. Genotyping of 82 SNPs from eight opioid genes (Table 3) was performed at the Rockefeller University Genomics Resource Center, using a 1536-plex Illumina Golden Gate Custom Panel (GS0013101-OPA), which is a modification of the "addiction array" that has been previously described (Hodgkinson et al., 2008; Levran et al., 2008). Data analysis was performed with *BeadStudio* v2.3.43 software (Illumina). Genotype data were visually inspected and filtered to include only SNPs with call rates > 90% and minor allele frequency (MAF) > 0.05.

2.4. Assessment of Percentage of European Ancestry

Assessment of ethnicity was initially based on self-reported family origin. Biographic ancestry scores were calculated using the program *Structure* v2.2 (Pritchard et al., 2000) with seven clusters based on 155 ancestry informative markers (AIMs). Each subject was anchored against 1050 samples from 51 populations represented in the Human Genome Diversity Cell Line Panel, as described (Ducci et al., 2009). To limit population stratification, the European ancestry contribution was arbitrarily set to a minimum of 70% to be included in the study.

2.5. Statistical analysis

In this study, a) *PLINK v1.9* (Purcell et al., 2007) was used for testing basic association and also for testing Hardy-Weinberg equilibrium (HWE); b) *Haploview v4.2* (Barrett et al., 2005) for estimation of pairwise linkage disequilibrium (LD); and c) *sumstat* (Hoh et al., 2001) for testing gene-gene interactions. For each group, to be conservative and only reject SNPs with strong deviation from HWE, a chi-square test with a critical limit for significance of p=0.0001 (0.01/n, with n = 82 SNPs evaluated) was used. Empirical significance levels for the basic association test, based on 100,000 permutations, were evaluated for the maximum chi-square test statistic from dominant/recessive/allelic tests, in two ways, nominally (p_0, for each SNP separately) and experiment-wise ($p_{corrected}$) for the largest result maximized over all SNPs.

SNPs with $p_{corrected}$ <0.05 were filtered for evaluation of potential gene-gene interactions. Interactions between a given significant SNP and all other SNPs were tested by conditioning on the three genotypes (AA, AB, BB) of the significant SNP and computing chi-squares for each of the resulting three datasets, with the maximum of the three chi-squares being taken as the relevant test statistic versus each other SNP (Wang et al., 2010). Associated significance levels were again evaluated in 100,000 permutation samples.

3. Results

3.1. Sample characteristics

Ancestry based on *Structure* analysis for all 795 subjects is shown in Figure 1. Based on self-report, 628 of the 795 subjects were identified as having European ancestry. Of these, 19 subjects had less than 70% European ancestry contribution leaving 609 subjects with genetically confirmed European ancestry for analyses. In addition, 12 subjects were excluded from analysis due to the low quantity and/or poor quality of the DNA (Table 1).

Drug use assessments from the KMSK lifetime scores is summarized in Table 2. A substantial number of subjects in all treatment groups had scores above the cut-point for alcohol dependence/addiction, from a low of 30% of healthy controls to a high of 72% of the *OD-MMT* group. Essentially no healthy controls used cocaine. In contrast, opioid dependent groups had a high percentage of subjects using cocaine, 65% of the subjects in *OD-HAT* and more than 85% of the *OD-MMT* subjects had KMSK scores above the cut point for cocaine dependence. Surprisingly, the *NOD* group had a considerable number of subjects, 33%, with moderate to heavy exposure to cocaine. The ten subjects (6%) in the *NOD* group with moderate to heavy opioid use can be expected due to the normal variance seen in the KMSK scale or any similar scale.

Comparison of the mean age and gender of the participants across treatment groups revealed several significant differences. The *OD* group (*MMT* + *HAT*) was older than both the *HC* and the *NOD* groups. The *HAT* subjects contained significantly more males than all other groups, 85%, compared to 65% for both the *MMT* and *NOD* groups and 56% for the *HC* group (Table 3).

Of the total 82 SNPs, eight were excluded based on MAF<0.05 (Table S1). Therefore, 74 SNPs from the eight opioid genes were analyzed for association to non-dependent opioid use and heroin dependence (Table 4 and Table S1). None of the SNPs significantly violated HWE. LD analysis of all SNPs, in the *HC* sample, revealed 19 SNP pairs that were in strong LD, $r^2 > 0.8$ (Figure S1). Five unique SNPs in four genes showed nominally (or point-wise) significant associations of genotype with non-dependent opioid use and/or heroin dependence with one SNP, *OPRD1* rs2236861 remaining significant after correction by permutation test (Table 5).

3.2. Healthy controls (HC) vs non-dependent opioid users (NOD)

In the comparison of the healthy controls to the non-dependent opioid group, four SNPs in three genes (*OPRD1,* rs2236861 and rs529520; *PENK*, rs2609998; and *OPRK1*, rs6473797) were found to have a nominally significant difference in their genotype frequency. One SNP, *OPRD1* rs2236861, also showed experiment-wise significance (OR = 0.032, 95% CI 0.002–0.540, $p_{corrected}$ =0.015). Conditional analysis for gene-gene interaction resulted in one SNP, rs2722897, in *PNOC,* with a maximum chi-square of 16.22 over the three genotypes at *OPRD1* rs2236861, which is experiment-wise significant with $p_{corrected}$ =0.041, whereas unconditional analysis with rs2722897 alone was non-significant. Thus, *PNOC* SNP rs2722897 exhibits significant results only when analyzed conditional on the genotypes of *OPRD1* SNP rs2236861 (Table 6). Detailed analysis demonstrated that for individuals with a

genotype of 'GG' at rs2236861, the 'GA' genotype at rs2722897 is associated with non-dependent opioid use, with computed odds ratio (OR) = 5.24 (95% CI, 2.04–13.45).

3.3. Healthy controls (HC) vs opioid dependent heroin users (OD)

When the healthy control group was compared to opioid dependent subjects (*MMT+HAT*), one variant in *PNOC*, rs2722897 was found to be nominally significant (p_0=0.022).

3.4. Non-dependent opioid users (NOD) vs opioid dependent users (OD)

In the comparison of non-dependent opioid users with the opioid dependent group, two SNPs showed nominal significance: *PENK* SNP, rs2609998 (p_0=0.006) and *OPRD1* SNP, rs2236861 (p_0=0.017).

The number of subjects with cocaine exposure above or below the KMSK cut-point in the *NOD* compared to *HC* is as a potential confounder for the genetic association findings. To verify this hypothesis, we tested the association between the number of subjects above the cut-point and the genotype frequency in each of the 4 significant SNPs (the experiment-wise significant SNP, rs2236861, and the 3 point-wise significant SNPs; rs529520, rs2609998, and rs6473797). Fisher's exact test showed no significant association (all p-values > 0.05; data not shown).

4. Discussion

The aim of this study was to identify variants in the eight opioid genes that play a role in the development of non-dependent heroin (or other opioid) use or heroin dependence, in a Dutch Caucasian population. Nominally significant differences between the groups were found for five unique SNPs in *OPRD1, OPRK1, PENK* and *PNOC.* All variants are from non-coding regions. The only result that remained significant after correction for multiple testing was the difference in the frequency of the *OPRD1* intronic SNP rs2236861 in the comparison between healthy controls to non-dependent opioid users. *Post-hoc* analysis revealed that this same SNP, rs2236861, also exhibited experiment-wise significance in a gene-gene interaction with *PNOC* SNP rs2722897.

OPRD1 encodes for the delta opioid receptor (DOP-r). DOP-r is known to be involved in a variety of neurological disorders and its activation reduces persistent pain and also depressive symptoms (e.g.Pradhan et al., 2011). Previous studies from our laboratory reported three *OPRD1* intronic SNPs (including rs2236861, rs3766951, and rs2236857, which are in moderate LD) to be associated with heroin addiction in Caucasian subjects (Levran et al., 2008). In a different study consisting of predominately Austrians, the *OPRD1* SNP rs2236861 was also shown to be significantly associated with opioid dependence (Beer et al., 2013). Finally, a large candidate gene association study in Australian subjects, comparing heroin dependent subjects to non-dependent controls, found 10 of 21 *OPRD1* SNPs to be nominally associated with heroin addiction, including SNP rs2236861 (Nelson et al., 2012). Of note, not all the studies reported associations in the same direction. In the present study, we found the *OPRD1* SNP rs2236861 'GG' genotype to be associated with non-dependent opioid use and not with heroin addiction.

PENK encodes the enkephalin peptides, which act primarily as agonists at the delta and the mu opioid receptors. In a family based association study, Xuei et al (2007) found evidence of association with opioid dependence of a three consecutive SNP haplotype block in *PENK*, rs1975285-rs2609998-rs2609997 (Xuei et al., 2007). Two of the SNPs from this haplotype block (rs1975285 and rs2609998) were included in our array with rs2609998 showing nominally significant associations for both dependent and non-dependent use liability.

The kappa opioid receptors (KOP-r), encoded by *OPRK1,* and their endogenous ligands, the dynorphins, encoded by *PDYN*, are involved in the modulation of reward from drugs of abuse, blunting dopaminergic surges (Butelman et al., 2012). In the present study the *OPRK1* SNP, rs6473797 was nominally significantly associated with non-dependent opioid use. In contrast, an earlier study from our laboratory found SNP rs6473797 to be associated with heroin addiction in Caucasian subjects (Levran et al., 2008), not what was found in the current study.

Nociceptin/orphanin FQ receptor (NOP-r), encoded by *OPRL1*, plays a role in regulating behavioral responses and tolerance to morphine through its interaction with its endogenous ligand nociceptin/orphanin FQ (N/OFQ), the gene product of *PNOC* (Clarke et al., 2003). N/OFQ reduces the rewarding properties of addictive drugs. We found one variant in *PNOC*, SNP rs2722897, to be significantly associated, point-wise, with heroin dependence. In addition, this SNP, through gene-gene interaction with another SNP (rs2236861) exhibited experiment-wise significance with non-dependent opioid use.

Interestingly, this study did not find any significant association for the studied SNPs in *OPRL1*, *OPRM1*, *PDYN* and *POMC.* In prior studies, we and others have shown a number of variants in these genes to be associated with heroin addiction (Bart et al., 2004; Bart et al., 2005; Clarke et al., 2009; Levran et al., 2008; Xuei et al., 2007; Yuferov et al., 2009; Zhang et al., 2009). It should be noted, however, that other studies also failed to find significant associations with SNPs from some of these same genes (e.g. Nelson et al., 2012). Exposed but not dependent opioid users remain an under studied group, and therefore it is challenging to point to prior studies that were not replicated by the current study.

The lack of power due to the relatively small sample size of our study may have limited detection of significant differences between groups.

5. Conclusion

Although most of the current study's findings were only nominally significant, they do add to the evidence that variants of the opioid genes play a role in heroin addiction (Levran et al., 2012; Reed et al., 2014). Future studies with greater statistical power are needed to corroborate the results and to evaluate the potential contribution of the findings for diagnosis and treatment. It is also clear from these data that the effects of these genes are likely to be rather limited in size and that genetic factors other than those related to the opioid system are also involved in non-dependent opioid use and heroin dependence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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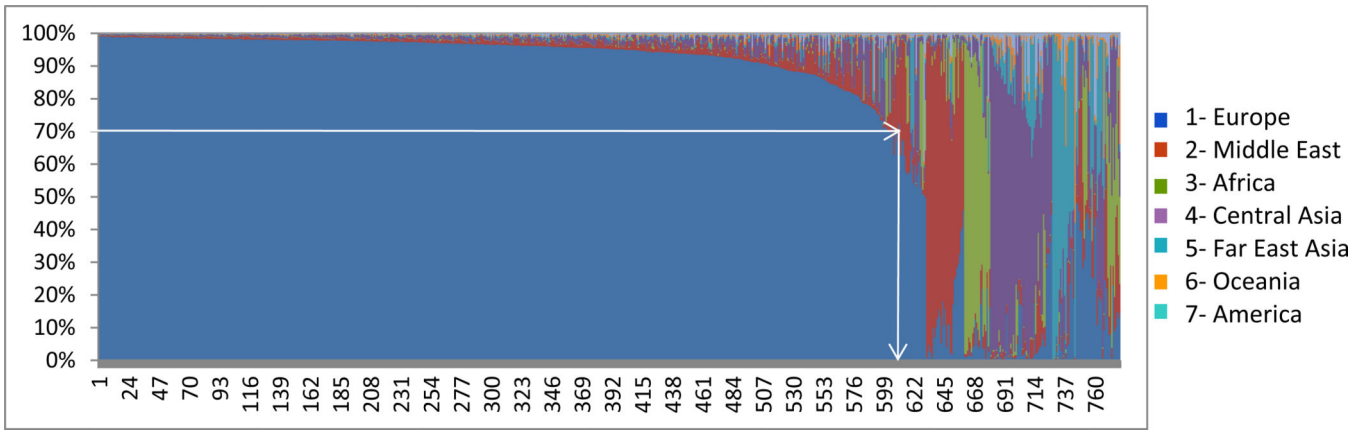

Figure 1:

Schematic representation of the individual admixture estimates of all subjects

Estimates are based on *STRUCTURE* analysis using $K = 7$. Each vertical line (x-axis) represents one subject and is displayed according to their predominant cluster contribution. The y-axis represents percentage of ancestry contribution. The clusters correspond to the geographical regions based on the HGDP sample.

Table 1.

Subject numbers by treatment group

Treatment Group	Recruited	Caucasian by self-report	Caucasian by AIMs	Excluded (low quality DNA)	Included in analysis
NOD	198	171	166	3	163
OD - MMT	204	150	144	1	143
OD - HAT	196	139	141	3	138
HC	197	168	158	5	153
Total	795	628	609	12	597

Table 2.

KMSK scores for heroin, cocaine, alcohol and nicotine

Treatment group	KMSK scale range	Heroin (9)* 0–13	Cocaine (11)* 0–16	Alcohol (11)* 0–13	Nicotine 0–13
NOD	Range	1–12	0–16	6–13	0–13
(n=163)	Mean ±SD	5.2 ± 2.1	8.5 ± 3.8	11.0 ± 1.6	10.2 ± 2.3
	Median	5	8	11	11
	Subjects over cut-point	10 (6%)	53 (33%)	111 (68%)	n/a
OD - MMT	Range	4–13	0–16	1–13	0–13
(n=143)	Mean ±SD	9.9 ± 2.2	12.8 ± 3.6	10.8 ± 3.0	9.7 ± 2.5
	Median	9	12	13	10
	Subjects over cut-point	127 (89%)	124 (87%)	103 (72%)	n/a
OD - HAT	Range	0–13	0–16	1–13	0–13
(n=138)	Mean ±SD	9.0 ± 2.2	10.9 ± 3.6	11.0 ± 3.0	9.9 ± 2.5
	Median	9	11	12	10
	Subjects over cut-point	94 (68%)	89 (65%)	94 (68%)	n/a
HC	Range	0–2	0–10	3–13	0–13
(n=153)	Mean ±SD	0.04 ± 0.27	0.7 ± 1.9	9.6 ± 2.1	6.0 ± 4.8
	Median	0	0	10	8
	Subjects over cut-point	0	0	47 (31%)	n/a

* Cut-point based on ROC analysis from prior studies (Kellogg et al., 2003)

NOD, non-dependent opioid user; *OD - MMT*, opioid dependent in methadone maintenance treatment; *OD - HAT*, opioid dependent in heroin-assisted treatment; *HC*, healthy control; SD, standard deviation

Table 3.

Subject demographics

A. Age and gender by treatment group

Treatment Group	Mean age (SD)	Male	Female
NOD	40.1 (9.0)	65%	35%
OD - MMT	43.5 (6.9	65%	35%
OD - HAT	43.4 (7.3)	85%	15%
HC	39.0 (10.4)	56%	44%

B. Comparison of age and gender between groups

	Age	Gender
Comparison	***p*-value***	***p*-value***
NOD vs *MMT*	**0.0011**	1
NOD vs *HAT*	**0.0017**	**0.0001**
NOD vs *HC*	0.2929	0.1477
MMT vs *HAT*	0.8921	**0.0001**
MMT vs *HC*	< **0.0001**	0.1477
HAT vs *HC*	< **0.0001**	< **0.0001**

NOD, non-dependent opioid user; *OD*, opioid dependent user; *MMT*, methadone maintenance treatment; *HAT*, heroin-assisted treatment; *HC*, healthy control; SD, standard deviation

** p*-values are FDR corrected

Table 4.

Opioid genes analyzed

Gene Symbol	Receptors
OPRD1	opioid receptor, delta
OPRK1	opioid receptor, kappa
OPRL1	opioid-like receptor 1
OPRM1	opioid receptor, mu
Gene Symbol	**Ligands**
PENK	proenkephalin
PDYN	prodynorphin
PNOC	prepronociceptin
POMC	proopiomelanocortin

Table 5.

Most significant associations

Group comparison	Gene	SNP	Chr	position	Alleles	Location	Test	p_0	$p_{corrected}$	OR (95% CI)
HC vs *NOD*	*OPRD1*	rs2236861	1	29139756	G/A	intronic	Rec	**0.0002**	**0.015**	0.032 (0.002–0.540)
	OPRD1	rs529520	1	29174946	C/A	intronic	Allelic	0.045	0.878	0.688 (0.503–0.942)
	PENK	rs2609998	8	57360034	C/T	5' upstream	Rec	0.017	0.631	2.688 (1.244–5.808)
	OPRK1	rs6473797	8	54152982	A/G	intronic	Rec	0.029	0.795	5.464 (1.191–25.067)
HC vs *OD*	*PNOC*	rs2722897	8	28173197	G/A	5' upstream	Dom	0.022	0.790	2.011 (1.138–3.555)
NOD vs *OD*	*PENK*	rs2609998	8	57360034	C/T	5' upstream	Rec	0.006	0.282	0.392 (0.208–0.737)
	OPRD1	rs2236861	1	29139756	G/A	intronic	Rec	0.017	0.557	15.22 (0.9–258.84)

Associations with $p_0 < 0.05$ are shown

NOD, non-dependent opioid users; *OD*, opioid dependent users; *HC*, healthy controls; Dom, dominant; Rec, recessive; OR, odds-ratio; CI, confidence interval; SNP, single nucleotide polymorphism; Chr, chromosome; p_0, nominal *p*-value.; $p_{corrected}$, *p*-value corrected by permutation test (n=100,000)

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Table 6.

Interaction of *PNOC* SNP rs2722897 and *OPRD1* SNP rs2236861 when non-dependent opioid users are compared to healthy controls

		rs2722897 genotype			
rs2236861 genotype		**AA***	**GA**	**GG**	**chi-square**
AA	*NOD*	0	0	0	
	HC	0	0	13	
GA	*NOD*	1	5	61	
	HC	1	10	44	
	OR (95% CI)	--	0.36 (0.12–1.13)	2.77 (0.89–8.68)	3.28
GG	*NOD*	0	27	67	
	HC	1	6	78	
	OR (95% CI)	--	5.24 (2.04–13.45)	0.19 (0.07–0.49)	16.22**

*Not used in calculation of OR owing to small number of observations

**$p_{corrected} = 0.041$

NOD, non-dependent opioid user; *HC*, healthy controls; OR, odds ratio; CI, confidence interval