Research Article

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Pharmacogenomics

VMAT2 gene (*SLC18A2*) variants associated with a greater risk for developing opioid dependence

Matthew Randesi*[,1], Wim van den Brink[2], Orna Levran[1], Peter Blanken[2,3], Jan M van Ree[4], Jurg Ott[5] & Mary Jeanne Kreek[1]

[1]Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

[2]Department of Psychiatry, Amsterdam Institute for Addiction Research, Academic Medical Center, University of Amsterdam, PO Box 22660, 1100 DD Amsterdam, The Netherlands

[3]Parnassia Addiction Research Centre (PARC, Brijder Addiction Treatment), PO Box 53002, 2505 AA The Hague, The Netherlands

[4]Rudolf Magnus Brain Center, University Medical Center Utrecht, Utrecht University, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

[5]Laboratory of Statistical Genetics, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

*Author for correspondence: randesm@rockefeller.edu

Aim: To determine if selected serotonergic and noradrenergic gene variants are associated with heroin addiction. **Subjects & methods:** A total of 126 variants in 19 genes in subjects with Dutch European ancestry from The Netherlands. Subjects included 281 opioid-dependent volunteers in methadone maintenance or heroin-assisted treatment, 163 opioid-exposed but not opioid-dependent volunteers who have been using illicit opioids but never became opioid-dependent and 153 healthy controls. **Results:** Nominal associations were indicated for 20 variants in six genes including an experiment-wise significant association from the combined effect of three *SLC18A2* SNPs (rs363332, rs363334 and rs363338) with heroin dependence (p_{final} = 0.047). **C onclusion:** Further studies are warranted to confirm and elucidate the role of these variants in the vulnerability to opioid addiction.

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Opioid addiction, like all addictions is a chronic, relapsing brain disease, has risen dramatically over the past 20 years and presents a major health and social problem worldwide. One reason for this rise has been the misuse of prescription opioids which can lead to dependence and often leads to heroin addiction.

Heroin addiction is a complex disease with a substantial genetic component. Studies have found association of numerous gene variants with heroin addiction, indicating that there may be many variants in diverse pathways contributing to the phenotype [1–9]. These include gene variants in mu (μ), kappa (κ) and delta (δ) opioid receptors, dopamine receptors D2 and D4, serotonin receptor 1B, FK506-binding protein 51, GABA receptor subunit γ-2, catechol-O-methyltransferase, proenkephalin, tryptophan hydroxylase 2 and brain-derived neurotrophic factor. Although the opioid and the dopaminergic systems are often thought to be the most common pathways for the reinforcing properties of drugs, other neurotransmitter pathways are undoubtedly involved, including the GABAergic, cholinergic and glutamatergic systems, as well as genes involved in stress-responsivity [10]. The current report focuses on the serotonergic and noradrenergic systems, and we hypothesize that gene variants in these systems may cause perturbations contributing to the vulnerability to opioid addiction.

In the brain, serotonin (5-HT), a monoamine neurotransmitter, is primarily produced by neurons of the raphe nuclei. Serotonin plays a critical role in the maintenance of synaptic plasticity, motivational and reinforcement processes, as well as for memory and learning. The serotonergic system has been implicated in several neuropsychiatric conditions [11]. Drugs of abuse – including heroin – produce acute changes in serotonin activity. Heroin addicts show a long period of reduced sensitivity of serotonin receptors even after drug cessation [12]. Serotonergic signaling is mediated via several G-protein-coupled receptors and an ion channel (the 5-HT3 receptor) that activate

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Table 1. Subject characteristics.

Treatment group	Subjects recruited (N_R)	Caucasian (self-report)	Caucasian (AIMs)	Excluded (DNA low quality)	Subjects analyzed (N_A)	Mean age ± SD	% Male
OD	400	289	285	4	281	43 ± 8	75
NOD	198	171	166	3	163	40 ± 9	65
HC	197	168	158	5	153	39 ± 10	56
Total	795	628	609	12	597	42 ± 10	68

AIM: Ancestry informative marker; HC: Healthy control; NOD: Exposed but not opioid dependent; OD: Opioid dependence; SD: Standard deviation.

the mesolimbic reward circuitry. In this study, we examined polymorphisms in genes encoding seven serotonergic receptors including: *HTR1A*, *HTR1B*, *HTR2A*, *HTR2B*, *HTR3A*, *HTR3B* and *HTR4*. Variants in six additional serotonergic genes were also studied including: *SLC6A4*, *SLC6A7*, *SLC18A2* and *S100A10* that is involved in regulating serotonin signaling, as well as two enzymes involved in serotonin biosynthesis: *TPH1* and *TPH2*.

The noradrenergic system is involved in various brain functions that are relevant to addiction including arousal, mood and learning, memory and stress response [13,14]. The noradrenergic system can also impact the hypothalamic–pituitary–adrenal stress axis [15]. During opiate withdrawal, norepinephrine (NE) neurons are strongly activated [16]. Activation of the central noradrenergic system influences the stress-induced re-instatement of drug-seeking behavior in animal models [17]. There is evidence in humans that stress responsivity has a substantial effect on drug relapse [18]. The current study examined variants in five noradrenergic receptors genes: *ADRA1A*, *ADRA2A*, *ADRA2B*, *ADRA2C* and *ADRB2*, as well as *SLC6A2*.

Several studies have evaluated the association between polymorphisms in both the noradrenergic and serotonergic genes and drug addiction. Associations of variants in the serotonin pathway with opioid dependence (OD) have been reported [19,20]. Several studies of OD that included serotonergic and noradrenergic genes were previously performed by our laboratory in subjects from varying ethnic groups [5,7,8,21]. These include association of OD with *HTR3B* SNPs rs3758987 and rs11606194, as well as with *HTR1B* SNP rs6297, in subjects with European ancestry. In African–Americans, interaction between SNPs in *TPH1*/*TPH2* has been reported to be associated with OD [21].

This hypothesis-driven, case–control association study was designed to determine if variants in genes of the noradrenergic and serotonergic pathways contribute to the vulnerability to develop OD in Caucasian subjects, including an understudied group of exposed but not opioid dependent (NOD) subjects, as a second control group.

The current study is an extension of our previous findings on the genetic variants that contribute to NOD and OD, in which analysis focused on variants from other system genes [22,23].

Materials & methods

Subjects

Three subject groups were recruited in The Netherlands as previously described (Table 1) [22–25], including:

- OD, opioid dependent patients meeting DSM-IV criteria for OD (defined as daily, multiple self-administrations of a short-acting opiate) for at least 5 years and in methadone maintenance treatment or heroin-assisted treatment (N_R = 400, N_A = 281);
- NOD, subjects exposed to illicit opioids, but not opioid dependent who reported a lifetime history use of heroin or other nonprescribed opioids without ever developing dependence on opioids. Subjects must have used heroin (or other nonprescribed opioids) at least five-times, but less than 100-times, with the first use at least 2 years prior to recruitment and never entered treatment to reduce or stop their opioid use (N_R = 198, N_A = 163);
- HC, healthy controls without a history of any illicit opioid use and with no history of alcohol or drug dependence according to DSM-IV criteria (N_R = 197, N_A = 153).

All participants had to be at least 25 years of age. Recruitment was through advertisements in local media, as well as through personal contacts or referral by other volunteers (snowball sampling).

The Central Committee on Research Involving Human Subjects in The Netherlands (protocol number P04.0156C) approved the study of heroin-assisted and 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. All subjects signed informed consent for the study, including the genetics research.

Table 2. Gene list.

Symbol	Gene name	Alias	SNPs genotyped	SNPs analyzed
Serotonergic				
HTR1A	5-Hydroxytryptamine (serotonin) receptor 1A, G-protein-coupled	5-HT1A	3	3
HTR1B	5-Hydroxytryptamine (serotonin) receptor 1B, G-protein-coupled	5-HT1B	7	6
HTR2A	5-Hydroxytryptamine (serotonin) receptor 2A	5-HT2A	17	16
HTR2B	5-Hydroxytryptamine (serotonin) receptor 2B	5-HT2B	6	5
HTR3A	5-Hydroxytryptamine (serotonin) receptor 3A, G-protein-coupled	5-HT3A	6	3
HTR3B	5-Hydroxytryptamine (serotonin) receptor 3B, G-protein-coupled	5-HT3B	9	5
HTR4	5-Hydroxytryptamine (serotonin) receptor 4, G-protein-coupled	5-HT4	4	3
S100A10	S100 Calcium binding protein A10	P11	4	4
SLC18A2	Solute carrier family 18, member A2 (vesicular monoamine)	VMAT2	12	9
SLC6A4	Solute carrier family 6, member 4, (serotonin transporter)	SERT	11	9
SLC6A7	Solute carrier family 6, member 7 (L-proline transporter)	PROT	7	5
TPH1	Tryptophan hydroxylase 1	TPRH	7	7
TPH2	Tryptophan hydroxylase 2	ADHD7	18	15
Adrenergic				
ADRA1A	Adrenoceptor α 1A	ADRA1C	16	13
ADRA2A	Adrenoceptor α 2A	ADRA2	3	3
ADRA2B	Adrenoceptor α 2B	ADRA2L1	3	2
ADRA2C	Adrenoceptor α 2C	ADRA2L2	4	4
ADRB2	Adrenoceptor β 2	ADRB2R	3	3
SLC6A2	Solute carrier family 6, member 2 (noradrenaline transporter)	NET1	12	11
Total			152	126

Socio-demographic & drug use assessment

All subjects were interviewed by trained clinical investigators. Standard questionnaires were used for collection of data on age, gender and country of origin. The DSM-IV was used for the diagnosis of OD.

Additionally, the Kreek–McHugh–Schluger–Kellogg (KMSK) scale [26], a rapid and quantitative instrument to assess a subject's exposure to opioids, cocaine, alcohol and nicotine was used to assess the frequency, amount and duration of exposure to each substance during the subject's period of greatest use (lifetime score) was administered in The Netherlands. Kreek–McHugh–Schluger–Kellogg assessments have previously been evaluated using receiver operator characteristics analysis for the optimal cut-point score for alcohol, cocaine and opiate dependence/addiction diagnoses using an ethnically diverse population [26]. The originally reported cut-points were used in the present study.

Genotyping

Genomic DNA was isolated from the blood specimens of all subjects by standard methods. Genotyping of 152 SNPs in 19 genes (Table 2 & Supplementary Table 1) was performed using a custom Illumina Golden Gate Panel (GS0013101-OPA; Illumina, Inc., CA, USA) [7], a modification of the 'addiction array' described previously [27]. Data analysis was performed using BeadStudio v2.3.43 (Illumina, Inc.). Clustered genotype data were manually inspected for quality. Only SNPs with good separation of clusters, call rates >90% and minor allele frequency (MAF) >0.05 were used for association analyses.

Assessment of percentage of European ancestry

Ethnicity was initially assigned based on self-reported family origin data with 628 self-identified Caucasian subjects, as previously described [22]. Based on 155 ancestry informative markers (AIMs), the fraction of genetic affiliation of the individual in each of the seven clusters was calculated using Structure v2.2 [28]. Each subject was anchored against 1051 samples from 51 worldwide populations represented in the Human Genome Diversity Cell Line Panel, as described [29]. For the current study the inclusion criteria was set to 70% or greater European ancestry contribution estimate to minimize population stratification, as described previously [22].

Statistical analyses

Using Plink v1.9 [30] we computed p-values for deviations from Hardy–Weinberg equilibrium (HWE). To be conservative and only reject SNPs with strong deviation from HWE, a χ^2 test with a critical limit for significance of $p \leq 0.01/n$, where n = number of SNPs evaluated, was used in healthy controls. Pairwise linkage disequilibrium (LD) was estimated using Haploview v4.2 [31]. Case–control association analyses were performed in two ways; for each SNP separately and for a combination effect of multiple SNPs.

Single SNP analysis

For each SNP, a maximum test was applied as follows. Consider a 2 × 3 contingency table with rows corresponding to cases and controls and the three columns representing the three genotypes, 1/1, 1/2 and 2/2, at a given SNP, where the '1' allele is the minor allele. For dominant action of the '1' allele, columns 1 and 2 (genotypes 1/1 and 1/2) are collapsed into a single column, resulting in a 2 × 2 table, for which χ^2 test is computed. Analogously, for recessive action, columns 2 and 3 are collapsed, leading to another 2 × 2 table with its χ^2 value. The larger of the two χ^2 represents the test statistic, t, for the given SNP, while $t_{\max}$ is the largest test statistic over all SNPs. Empirical significance levels (p-values) were obtained via permutation sampling by randomly permuting the labels case and control. In each of the resulting permuted datasets, the same test statistics were computed for each SNP and over all SNPs. For each SNP, a nominal significance level, p_0, was obtained as the proportion of randomization samples with a value of t at least as large as the observed t, and a significance level corrected for testing multiple SNPs – p – was given as the proportion of randomization samples with $t_{\max}$ at least as large as in the observed data. These significance levels were based on a total of 100,000 permutation samples.

Gene-based analysis via scan statistics

Burden tests are a common approach to capture the joint association effect of multiple contiguous SNPs [32,33]. To obtain accurate significance levels with such a procedure, we implemented a burden test via scan statistics in the following manner. Consider a window of a certain size, n (number of contiguous SNPs), and within this window we compute some test statistic, h. The window is moved across all the studied SNPs within a chromosome, one by one, and at each step h is recalculated. The largest value of h so obtained is called the scan statistic of size n [34]. In our implementation [35], we work with windows of sizes n = 1, 2, ... n_{max}, where $n_{\max}$ is a suitable upper limit. For each n, the scan statistic is computed and an associated p-value, p_n, is obtained from permutation analysis. The smallest such p is then taken as the overall test statistic, for which an associated significance level, p_{final}, is obtained from the permutation samples.

The burden test statistic is computed as follows. For a given window of size n, for each of cases and controls, we tally the number, N_1, of individuals with a '1' allele (at any of the n SNPs in the given window) and the number N_0 of individuals without a '1' allele, where '1' is the minor allele and '2' is the major allele. Thus, each window furnishes a 2 × 2 table with rows corresponding to cases and controls, and columns referring to '1' and not '1' individuals. We define a one-sided test statistic – g – in the expectation that cases tend to more often carry a '1' allele than controls. Thus, with χ^2 obtained for the 2 × 2 table, $g = \sqrt{(\chi^2)}$ if more cases than controls carry a '1' allele, and $g = -\sqrt{(\chi^2)}$ if more controls than cases carry a '1' allele. We chose $n_{max} = 3$ as the upper window size in scan statistics.

Results

Of the 152 SNPs genotyped in the selected genes, 19 variants were excluded due to the MAF < 0.05 and seven SNPs were excluded due to a call rate of less than 90%. The remaining 126 SNPs were analyzed for association with exposed but not opioid dependent use (NOD) and OD (Supplementary Table 1). No SNP showed significant deviation from HWE in the control sample. LD analysis of the control sample revealed eight SNP pairs and one SNP triplet in complete LD ($r^2 = 1.0$) as well as ten SNP pairs and two SNP triplets in strong linkage ($r^2 > 0.7$) (Supplementary Figure 1).

Sample characteristics

Verification of the ancestry of all subjects using Structure analysis of the ancestry informative markers resulted in the exclusion of 19 subjects who described themselves as Caucasian but with a European ancestry contribution estimate of less than 70%. No evidence of population substructure was found within each study group, as described

Table 3. Locus-specific statistic: maximum χ^2 for dominant and recessive models.

Comparison	SNP	Gene	Chr	Location	Model	Alleles	p_0	p	OR (95% CI)
HC vs NOD	rs363271	*SLC18A2*	10	Intron	Rec	T > G	0.034	0.575	6.87 (0.98–56.47)
	rs3758987	*HTR3B*	11	Upstream 2KB	Rec	A > G	0.020	0.806	3.06 (1.22–7.66)
	rs11606194			Intron	Dom	T > C	0.031	0.984	2.02 (1.08–3.77)
	rs2036108	*ADRA1A*	8	Intron	Rec	G > A	0.032	0.852	4.60 (1.15–18.40)
	rs2291776			Intron	Dom	G > A	0.046	0.995	2.04 (1.04–4.00)
NOD vs OD	rs3758987	*HTR3B*	11	Upstream 2KB	Dom	A > G	0.005	0.389	0.61 (0.41–0.90)
	rs1176744			Tyr129Ser	Dom	T > G	0.026	0.883	0.62 (0.42–0.92)
	rs11606194			Intron	Dom	T > C	0.040	0.996	0.56 (0.34–0.95)
	rs1150226	*HTR3A*	11	Upstream	Dom	C > T	0.006	0.588	0.46 (0.27–0.78)
	rs2276302			Intron	Rec	A > G	0.042	0.980	0.47 (0.25–0.89)
	rs363338	*SLC18A2*	10	Intron	Dom	T > C	0.031	0.907	1.63 (1.10–2.40)
HC vs OD	rs472151	*ADRA1A*	8	Intron	Rec	G > A	0.003	0.194	2.38 (1.37–4.16)
	rs10503800			Intron	Rec	C > A	0.031	0.926	2.83 (1.11–7.26)
	rs2036109†			Intron	Dom	G > T	0.020	0.830	0.59 (0.39–0.88)
	rs2055195†			Intron	Dom	G > A	0.031	0.943	0.59 (0.38–0.91)
	rs3785143	*SLC6A2*	16	Intron	Dom	C > T	0.016	0.794	2.18 (1.17–4.05)
	rs6587640	*S100A10*	1	3′ UTR	Rec	C > T	0.032	0.940	2.15 (1.11–4.16)
	rs3782025	*HTR3B*	11	Intron	Dom	T > C	0.040	0.975	1.67 (1.09–2.37)
HC + NOD vs OD	rs363338‡	*SLC18A2*	10	Intron	Dom	T > C	0.009	0.589	1.59 (1.16–2.20)
	rs363334‡			Intron	Dom	C > G	0.021	0.846	1.53 (1.11–2.12)
	rs363332‡			Intron	Dom	G > A	0.026	0.901	1.51 (1.09–2.10)
	rs2036109§	*ADRA1A*	8	Intron	Dom	G > T	0.018	0.792	0.65 (0.47–0.89)
	rs2055195§			Intron	Dom	G > A	0.021	0.830	0.63 (0.44–0.90)
	rs472151			Intron	Rec	G > A	0.028	0.889	1.67 (1.12–2.50)
	rs3791153#	*S100A10*	1	Intron	Rec	T > C	0.047	0.977	1.87 (1.09–3.23)
	rs12083193#			Intron	Rec	G > C	0.047	0.977	1.87 (1.09–3.23)
	rs1176744	*HTR3B*	11	Tyr129Ser	Rec	T > G	0.040	0.960	0.48 (0.25–0.91)
	rs1150226	*HTR3A*	11	Upstream	Dom	C > T	0.031	0.982	0.58 (0.36–0.94)
	rs3785143	*SLC6A2*	16	Intron	Dom	C > T	0.038	0.689	1.911.20–3.03)

Alleles given as major > minor in the HC.
† Represent SNPs in strong linkage disequilibrium ($r^2 > 0.7$).
‡ Represent SNPs in strong linkage disequilibrium ($r^2 > 0.7$).
§ Represent SNPs in strong linkage disequilibrium ($r^2 > 0.7$).
# Represent SNPs in complete LD ($r^2 = 1.0$).
Chr: Chromosome; HC: Healthy controls; LD: Linkage disequilibrium; NOD: Exposed but not opioid dependent; OD: Opioid dependence; OR: Odds ratio; p: Corrected significance level of given statistic; p_0: Nominal significance level of given statistic; Stat: Test statistic; Sum: Sum of largest n test statistics.

previously [22]. An additional 12 subjects were excluded from the association analyses due to low quantity or poor quality of the DNA sample (Table 1). The remaining 597 subjects were included in the association analyses.

Single SNP analysis

Four independent group comparisons were performed under both dominant and recessive models of inheritance: HC versus NOD; NOD versus OD; HC versus OD and HC + NOD versus OD (Table 3). In total, 20 variants in six genes were shown to have nominally significant association of genotype with OD or NOD; including, variants in two serotonin receptor genes (*HTR3A* and *HTR3B)*, the vesicular monoamine transporter 2 gene *(SLC18A2)*, the S100 calcium binding protein A10 gene (*S100A10*), adrenergic receptor α-1A gene *(ADRA1A)* and the noradrenaline transporter gene (*SLC6A2*).

HC versus NOD

Individual SNP analysis revealed nominally significant associations for five variants in three genes, where the minor alleles were all associated with greater risk. Two intronic variants in *ADRA1A* – rs2036108 and rs2291776 –

showed significant association (p_0 = 0.032 and p_0 = 0.046, respectively). Two SNPs in *HTR3B*, the upstream SNP rs3758987 and the intronic SNP rs11606194, displayed significant association (p_0 = 0.020 and p_0 = 0.031, respectively). The *SLC18A2* intronic variant rs363271 showed significant association (p_0 = 0.034).

NOD versus OD

We found nominally significant association of genotype with OD for six SNPs in three genes, where the minor allele had a protective effect, except where noted. Three SNPs in *HTR3B*, displayed significant associations, including: rs3758987, rs11606194 and a nonsynonymous SNP, rs1176744 (Tyr129Ser; p_0 = 0.005, p_0 = 0.040 and p_0 = 0.026, respectively). Two SNPs in *HTR3A*, the upstream variant rs1150226 and intronic variant rs2276302, were found to be significantly associated (p_0 = 0.006 and p_0 = 0.042, respectively). Intronic SNP rs363338 in *SLC18A2* showed significant association (p_0 = 0.031), where in contrast the minor allele was associated with a risk effect.

HC versus OD

Seven SNPs in four genes showed nominal significance, where the minor allele contributed to a risk effect, unless otherwise noted. We found significant associations of four intronic SNPs in *ADRA1A*; including, rs475151, rs10503800, rs2036109 and rs2055195 (p_0 = 0.003, p_0 = 0.031, p_0 = 0.020 and p_0 = 0.031, respectively). The minor alleles for SNPs, rs2036109 and rs2055195, provided a protective effect. SNPs rs2036109 and rs2055195 are also in strong LD (r^2 = 0.75). Three additional SNPs were found to be significantly associated with OD: an intronic variant in *SLC6A2*, rs3785143 (p_0 = 0.016), 3' UTR variant rs6587640 in the calcium binding protein gene *S100A10* (p_0 = 0.032) and the *HTR3B* intronic variant rs3782025 (p_0 = 0.040).

HC + NOD versus OD

Single SNP analysis revealed 11 SNPs in six genes with nominally significant association of genotype with OD, where the minor alleles showed a risk effect, unless otherwise noted. Three intronic *SLC18A2* SNPs, rs363338, rs363334 and rs363332 displayed significant signals (p_0 = 0.009, 0.021 and 0.026, respectively). Three intronic *ADRA1A* SNPs, rs2036109, rs2055195 and rs472151 also showed significant association (p_0 = 0.018, 0.021 and 0.028, respectively). Consistent with the finding for the comparison HC versus OD, having a minor allele for SNPs rs2036109 and rs2055195 would provide protection to individuals. Two intronic *S100A10* SNPs, rs3791153 and rs12083193 (both with p_0 = 0.047) were also found to be significant. Finally, three SNPs: rs1150226 in *HTR3A*, rs1176744 in *HTR3B* and rs3785146 in *SLC6A2* were found to have a significant association of genotype with OD (p_0 = 0.031, p_0 = 0.040 and p_0 = 0.038, respectively). The minor alleles of SNPs rs1150226 and rs1176744 provided greater protection.

Gene-based analysis via scan statistics

For each of the four comparisons, Table 4 shows scan statistics results with window sizes 1 to 3, where a window of size 1 refers to the best (most significant) SNP among all 126 SNPs analyzed in the sense that the given SNP exhibits the strongest difference between cases and controls for individuals carrying a minor allele. For each comparison, the overall significance level, p_{final}, was smaller than the significance level for single SNPs, p_0 (window length of one SNP), that is, burden test statistics comprising multiple contiguous SNPs appear more powerful than association tests based on individual SNPs. To obtain a result for the given comparison containing only SNPs in one gene, we carried out an analysis windows of sizes up to n_{max} = 3. The strongest effect was obtained for the comparison HC + NOD versus OD with a window length of three SNPs that consisted of the *SLC18A2* SNPs, rs363338, rs363334 and rs363332 (p = 0.022). The three variants had a combined effect which remained significant after correction for testing multiple window sizes and multiple SNPs for the given comparison, with p_{final} = 0.047. These SNPs (rs363332, rs363334 and rs363338) were found to be in strong LD, r^2 ranging from 0.78–0.98 (Figure 1).

Discussion

In this study, we analyzed 126 SNPs in 19 serotonergic and noradrenergic pathways genes for association with nondependent opioid use and/or heroin dependence. We found that 17 variants in six of the studied genes had nominally significant association of genotype with OD, and five variants in three genes had nominally significant association of genotype with NOD. The most significant result of the current study was revealed with a burden test that captures joint association effects of multiple contiguous SNPs. Specifically, for the comparison of HC + NOD versus OD using scan statistics, the strongest effect was found in *SLC18A2* and included SNPs rs363338, rs363334

Table 4. Significant associations by scan statistics.

Comparison	Gene	chr	L	SNP-1	SNP-2	SNP-3	BP-1	BP-L	scanstat	p	p_{final}
HC vs NOD	*HTR3B*	11	1	rs11606194			113,910,259	113,910,259	2.244	0.682	
			2	rs3758987	rs11606194		113,904,553	113,910,259	2.658	0.544	
			3	rs3758987	rs11606194	rs1176744	113,904,553	113,932,306	3.117	0.275	0.468
NOD vs OD	*SLC18A2*	10	1	rs363338			117,249,878	117,249,878	2.463	0.458	
			2	rs363334	rs363338		117,245,484	117,249,878	3.310	0.195	
			3	rs363332	rs363334	rs363338	117,243,156	117,249,878	3.934	0.072	0.139
HC vs OD	*SLC6A2*	16	1	rs3785143			55,661,194	55,661,194	2.588	0.359	
			2	rs363334	rs363338		117,245,484	117,249,878	2.946	0.365	
			3	rs363332	rs363334	rs363338	117,243,156	117,249,878	3.539	0.149	0.270
HC + NOD vs OD	*SLC18A2*	10	1	rs363338			117,249,878	117,249,878	2.815	0.205	
			2	rs363334	rs363338		117,245,484	117,249,878	3.801	0.075	
			3	rs363332	rs363334	rs363338	117,243,156	117,249,878	4.540	0.022	0.047

BP-1: Basepair position of SNP-1; BP-L: Basepair position of last SNP in scan window; chr: Chromosome; HC: Healthy controls; L: Length of scan statistic (number of SNPs); NOD: Exposed but not opioid dependent; OD: Opioid dependence; p: Significance level obtained for given scan statistic, corrected for testing multiple SNPs; p_{final}: Significance level corrected for testing multiple SNPs and multiple window sizes; scanstat: Scan statistic for given window size; SNP-1: First SNP in given window; SNP-2: Second SNP in given window; SNP-3: Third SNP in given window.

and rs363332. The three SNPs had a combined effect that withstood correction for testing multiple SNPs and multiple window sizes.

Serotoninergic genes

The *VMAT2* gene or *SLC18A2*: monoamine neurotransmitters such as serotonin, dopamine, epinephrine and norepinephrine are packaged into secretory vesicles by two different 12 transmembrane proteins known as VMATs, or vesicular monoamine transporters, which are part of a larger family of proteins called solute carriers (SLC) [36]. VMAT2, whose gene variants were indicated in the current study, is mainly confined to the central nervous system where it packages neurotransmitters into synaptic vesicles [37]. It is an antiporter that uses a pH gradient to allow two protons to exit while simultaneously translocating a single monoamine molecule, like serotonin, into the vesicle [38]. The capacity of these transporters can be upwards of 20,000 molecules, orders of magnitude greater than cytosolic levels [39]. Three intronic *SLC18A2* SNPs (rs363338, rs363334 and rs363332) were implicated in the present study to be associated with OD, two of which (rs363332 and rs363334) are in remarkedly high LD and the third in moderate LD (rs363338). To the best of our knowledge, this is the first report of association of these SNPs with OD. In a prior study from this laboratory using an entirely separate study group [40] three different *SLC18A2* SNPs (rs363271, rs2244249 and rs363276) were found to offer protection from OD with or without cocaine comorbidity, of which one (rs363271) was also indicated in the present study, where the association found provided more risk for nondependent opioid use.

Several SNPs in *S100A10* were indicated in the present study. The protein product of *S100A10*, p11 was found to be an important factor mediating antidepressant responses and depression-like states in mice [41]. A study of cannabis dependence found a possible gene-cannabis interaction for *S100A10* rs72993629 [42], a variant which was not included in the present study. Of the three *S100A10* variants found to be associated with OD in the present study, the intronic SNPs rs3791153 and rs12083193 were previously reported by our laboratory using an entirely different study sample [40] to convey protection from cocaine dependence, where the minor allele provided protection from OD. For both studies, we found SNPs rs3791153 and rs12083193 to be in complete LD in the control sample. The third *S100A10* SNP indicated in the present study, rs6587640, is reported for the first time to be associated with drug addiction.

Variants in two serotonin receptor genes, *HTR3A* and *HTR3B*, were indicated in the present study. The two receptor genes reside in close proximity to one another on chromosome 11 [43]. In a multisite study, the A allele of an intronic *HTR3B* SNP, rs3782025, was associated with alcohol dependence in a Finnish sample [44]. Another study found that an *HTR3B* missense SNP, rs1176744 (Tyr129Ser), predicted alcohol dependence in an African–American population [45]. In an earlier study of an entirely different group of volunteers, our laboratory found a nominally significant association of the C allele of *HTR3B* rs3758987 with OD [7]. In a different study, Yang

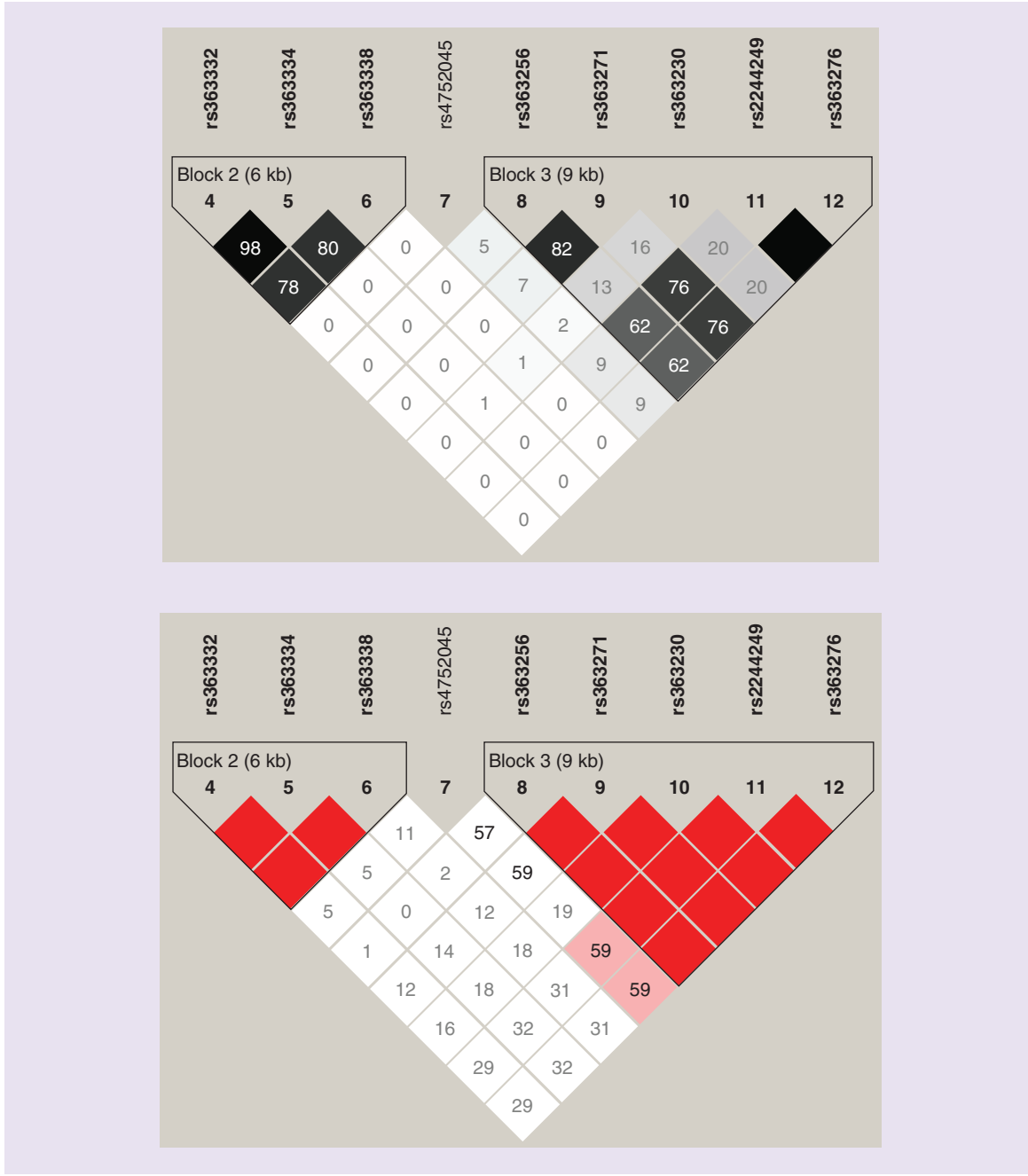

Figure 1. Linkage disequilibrium of *SLC18A2* SNPs. The pairwise correlation between SNPs measured as r^2 (top panel) and D′ (bottom panel). The values are shown (×100) in each box. The color scheme indicates the magnitude of the value where the darker the color indicates the greater linkage disequilibrium. When the linkage disequilibriumvalue is equal to 1.0 the box is empty.

et al. [46] reported a haplotype of six *HTR3A* SNPs with an experiment-wise significant association with nicotine dependence in an African–American sample. Two of the SNPs in the haplotype, rs1150226 and rs2276302, were found associated with OD in the present study.

Noradrenergic genes

We found six intronic variants in adrenoceptor α-1A gene (*ADRA1A*) to have significant associations; including rs2036109, rs2055195, rs10503800 and rs472151, which were found to be associated with OD and rs2036108

and rs2291776, found to be associated with NOD. SNP rs2036109 was previously associated with bipolar disorder in a family-based study [47]. In an earlier study of an entirely different cohort, we had shown that the three *ADRA1A* variants – rs10503800, rs472151 and rs2291776 – were associated with OD, with or without concomitant cocaine dependence in a European–American population [40]. Two *ADRA1A* SNPs, rs2036108 found associated with NOD and rs2055195 found associated with OD, had not previously been reported to be associated with OD.

Additionally, the intronic *SLC6A2* SNP, rs3785143, that the current study found associated with OD with the minor T allele conveying greater risk or vulnerability to develop OD, had previously been reported to be associated (point-wise significant) with methylphenidate response in patients with attention deficit hyperactivity disorder (ADHD) [48], as well as in a 2008 study, that found the variant to be nominally associated with ADHD [49].

Limitations

The relatively small number of subjects in the current study may have limited detection of additional significant differences between groups. Further studies with greater statistical power are warranted to corroborate the results and to assess the clinical significance of the findings.

It is noteworthy; however, that although just one of the reported associations survived correction for multiple testing, many of the other results replicate earlier findings from our laboratory with entirely separate and distinct study populations.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:

www.futuremedicine.com/doi/suppl/10.2217/pgs-2018-0137

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Authors' contributions

MJ Kreek, JM van Ree and W van den Brink originally conceived and designed the study. MJ Kreek oversaw all aspects of the study, as the principal investigator. M Randesi oversaw sample preparation, data collection, analysis, interpretation and drafting the manuscript. J Ott performed all statistical analyses. O Levran oversaw array design, SNP selection, data analysis and interpretation. P Blanken ascertained study subjects.

All co-authors contributed to the content of manuscript, provided critical reviews and approved the final version of the manuscript.

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