Research Article

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Pharmacogenomics

Further evidence for the association of *GAL*, *GALR1* and *NPY1R* variants with opioid dependence

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Aim: Heroin addiction is a chronic, relapsing disease that has genetic and environmental, including drug-induced, contributions. Stress influences the development of addictions. This study was conducted to determine if variants in stress-related genes are associated with opioid dependence (OD). **Patients & methods:** One hundred and twenty variants in 26 genes were analyzed in 597 Dutch subjects. Patients included 281 OD in methadone maintenance with or without heroin-assisted treatment and 316 controls. **Results:** Twelve SNPs in seven genes showed a nominally significant association with OD. Experiment-wise significant associations ($p < 0.05$) were found for three SNP pairs, through an interaction effect: *NPY1R*/*GAL* rs4691910/rs1893679, *NPY1R*/*GAL* rs4691910/rs3136541 and *GALR1*/*GAL* rs9807208/rs3136541. **Conclusion:** This study lends more evidence to previous reports of association of stress-related variants with heroin dependence.

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Keywords: case–control association study • *GAL* • *GALR1* • *NPY1R* • opioid dependence

Heroin dependence is a chronic, relapsing brain disease. An individual's vulnerability to develop a drug addiction is influenced by environmental, genetic and drug-induced factors [1–3]. Stress and anxiety play a key role in both the initiation of and relapse in drug addiction. Identification of the factors 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 to determine the specific factors responsible. Requirements for any methodological sound case–control association study include subjects that have been very carefully phenotyped as well as a population of subjects that are ethnically homogeneous.

Stress is a critical risk factor affecting both the development of addictive disorders, by promoting drug seeking and excessive drug intake, and the relapse to addictive behaviors, when drug withdrawal can increase stress response, which increases reward-seeking behavior [3–6]. Stress exposure, as well as drugs of abuse, activate the hypothalamus-pituitary-adrenal axis causing the release of both corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus paraventricular nucleus [7]. CRH and AVP are subsequently transported to the anterior pituitary, where they stimulate adrenocorticotrophic hormone (ACTH) release, which in turn stimulates synthesis and release of glucocorticoids from the adrenal cortex. The activity of the hypothalamus–pituitary–adrenal axis is regulated by glucocorticoids through negative feedback [8].

Table 1. Subject characteristics.

Treatment group	Subjects recruited (N_R)	Caucasian (self-report)	Caucasian (AIMs)	Subjects excluded (DNA low quality)	Subjects analyzed (N_A)	Mean age ± SD	Female (%)
HC	197	168	158	5	153	39 ± 10	44
NOD	198	171	166	3	163	40 ± 9	35
OD	400	289	285	4	281	43 ± 8	25
Total	795	628	609	12	597	42 ± 10	32

AIM: Ancestry informative markers; HC: Healthy control; NOD: Not opioid dependent; N_R: Number recruited; OD: Opioid dependent; SD: Standard deviation.
Adapted with permission from [30].

During opioid withdrawal, norepinephrine neurons are strongly activated [9]. Activation of the central noradrenergic system influences the stress-induced reinstatement of drug-seeking behavior in animal models [10]. There is evidence in humans that stress responsivity has a substantial effect on relapse to drug use [11].

Several studies have looked for the association of SNPs in stress-related genes with affective disorders and addictions to specific drugs of abuse. One such study found that a variant in the glucocorticoid receptor gene (*NR3C1*) lowered cortisol response to psychosocial stress [12]. Another study found that variants in both *GAL* and its receptors (*GALR1, GALR2* and *GALR3*) conferred an increased risk of depression and anxiety in a population who had experienced childhood adversity [13]. Variants in neuropeptide Y receptor genes, *NPY2R* and *NPY5R*, were found to be associated with alcohol and cocaine dependence [14]. There are few previous studies from this laboratory and others that have reported an association between stress-related gene variants and heroin addiction [15–21].

We will look for instances of both monogenic and digenic associations. Instances of so-called digenic inheritance have been reported where two variants in combination are disease associated [22]. For example, a family has been described in which severe insulin resistance occurred only in the presence of two mutations, one each in two different genes [23]. More recently, digenic inheritance for myocardial infarction has been documented [24]. In the extreme, it is conceivable that two variants interact and lead to disease and neither of the two variants is disease-associated [25]. This principle of subdividing data on the basis of one variant and carrying out statistical tests in the resulting subdivisions for another variant had been applied successfully in early analyses of diabetes susceptibility [26] and was more recently formalized as a sequential procedure [27]. It can lead to 'chains' of variants when target SNPs are reused as test SNPs until a target SNP points back to the original test SNP or the chain ends with no more target SNPs showing $p < 0.50$. Digenic associations were previously reported for heroin dependence [28], where epistatic effects between variants of the kappa opioid receptor gene and A118G of the mu opioid receptor gene increased the susceptibility to addiction in an Indian population.

Here, we report the results of an association study of 120 variants in 27 stress-related genes to establish the role of these variants in opioid dependence in Caucasian subjects from the Netherlands.

Materials & methods

Subjects

This study is a continuation of our previous studies where three subject groups were recruited in the Netherlands as previously described in Table 1 [29–31]. Briefly, a total of 795 subjects, 30% females, were ascertained including:

- Healthy controls (HC) without a history of illicit opioid use and with no history of alcohol or drug dependence by Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria (number recruited [N_R] = 197; number analyzed [N_A] = 153);
- Nonopioid-dependent (NOD) subjects who used illicit opioids, but never became opioid dependent (OD). Subjects must have used heroin (or other nonprescribed opioids) at least five-times but not more 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 and N_A = 163). These two groups (HC and NOD) were combined and used as the control group;
- OD patients who met DSM-IV criteria for opioid dependence for at least 5 years and were in methadone maintenance treatment or heroin-assisted treatment at the time of recruitment (N_R = 400 and N_A = 281).

All participants were at least 25 years of age at the time of recruitment. Recruitment of the control group was through advertisements in local media, as well as through personal contacts or referral by other volunteers (snowball sampling), whereas recruitment of the OD group was through addiction treatment centers. All subjects

provided written informed consent for the study. The Central Committee on Research Involving Human Subjects in the Netherlands approved the study of heroin-assisted and methadone maintenance treatments and the human molecular genetics study for all study groups (protocol no. P04.0156C). Approval of the genetics study was also obtained from The Rockefeller University's Institutional Review Board.

All subjects were interviewed by trained clinical investigators. Collection of data on age, gender and country of origin was obtained with standard questionnaires. The Substance Use Disorder section of the computerized fully structured Composite International Diagnostic Interview was used to obtain DSM-IV substance dependence diagnoses [32].

Genotyping

Coded blood specimens were collected in the Netherlands and shipped to the Laboratory of the Biology of Addictive Diseases at The Rockefeller University (NY, USA), where the DNA was isolated by standard methods. The quality of the DNA was assessed by agarose gel electrophoresis and quantified with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). Genotyping of 143 SNPs from 27 stress-related genes (Tables 2 & 3) was performed with an Illumina® GoldenGate custom panel (GS0013101-OPA, Ilumina, CA, USA), a modification of the 'addiction array' that has been previously described [17,33]. Genotyping was performed at The Rockefeller University Genomics Resource Center and analyzed with BeadStudio v2.3.43 software (Illumina). Genotype data were visually inspected and filtered to include only SNPs with good separation of clusters, call rates greater than 90% and minor allele frequency (MAF) greater than 0.05 for the association analyses.

Assessment of percentage of European ancestry

Ethnicity was initially based on the subjects self-reported family origin with 628 self-identified Caucasian subjects (Table 1). Using 155 ancestry informative markers, each individual's biographic ancestry score was calculated using Structure v2.2 [34]. Thus, the ancestry of each subject was determined individually with reference to a panel of 1051 individuals from 51 populations represented in the Human Genome Diversity Cell Line Panel, as described [35]. For the current study, subjects with 70% or greater European ancestry contribution were included in the association analyses in order to limit population stratification.

Statistical analyses

Our previous studies [30,31] with this same cohort of subjects found very few genetic differences between the HC and NOD subject groups, therefore for this study we combined the HC and NOD groups and used this larger combined 'control' group (N_A = 316) for comparison to the OD subjects ('cases').

PLINK v1.9 was used to test for deviations from Hardy–Weinberg equilibrium. Haploview v4.2 [36] was used for the estimation of pairwise linkage disequilibrium (LD). Genetic case–control association analysis was carried out using two approaches: one variant at a time and testing for the combined effects of two variants. In the former approach, three different association tests were applied to each variant as follows:

1. The genotype test compares frequencies of the three genotypes between cases and controls and results in a Chi-square statistic with two degrees of freedom;
2. Developed some 10 years ago, a specific F-test [37] compares allele frequencies between cases and controls and does so by restricting the parameter space in a biologically meaningful manner;
3. Finally, a maximum test leads to the larger of two Chi-squares, one Chi-square assuming dominant and the other Chi-square assuming recessive inheritance of the minor allele.

As test statistics in 2 and 3 do not have known null distributions, empirical significance levels (p-values) were estimated based on 10,000 permutation samples (labels for case and control were randomly permuted), with the observed data being the first of these 10,000 samples, so that the smallest possible significance level was p = 1/10,000 = 0.0001. Resulting significance levels were obtained in two ways, that is, in a nominal manner (p_0) and adjusted for testing multiple SNPs (p).

In the second approach, we looked for instances of digenic inheritance in our data. First, we collected a set of N variants as 'test SNPs' with $p < 0.50$ in any of the three single-variant tests (corrected for multiple testing). For a given test SNP, the data (cases and controls) were then divided into three groups depending on the genotype at the test SNP, in other words, all individuals in group 1 had genotype A/A at the test SNP, group 2 individuals

Table 2. Variants genotyped.

SNP	Gene	Name	Chr.	Position	Alleles	MAF	Comment
rs2740204	*AVP*	Arginine vasopressin	20	3081820	[A/C]	0.40	
rs2282018			20	3084302	[T/C]	0.36	
rs3761249			20	3085715	[T/G]	0.10	
rs1587097	*AVPR1A*	Arginine vasopressin receptor 1A	12	63136464	[T/C]	0.12	
rs10784339			12	63144865	[G/C]	0.19	
rs11174811			12	63146695	[A/C]	0.16	
rs3803107			12	63147053	[T/C]	0.18	
rs1042615			12	63150428	[T/C]	0.43	
rs3021530			12	63151308	[A/C]	0.01	MAF <0.05
rs3021529			12	63151899	[T/C]	0.17	
rs10877969			12	63153458	[T/C]	0.16	
rs3759292			12	63153532	[T/C]	0.01	MAF <0.05
rs7294536			12	63154311	[A/G]	0.16	
rs10877970			12	63157373	[A/G]	0.16	
rs33933482	*AVPR1B*	Arginine vasopressin receptor 1B	1	206110066	[T/C]	0.12	
rs33976516			1	206110179	[G/C]	0.03	MAF <0.05
rs28632197			1	206110372	[A/G]	0.01	Call rate <90%
rs28536160			1	206117947	[C/T]	0.04	MAF <0.05
rs3846658	*CARTPT*	CART prepropeptide	5	71714320	[A/T]	0.03	MAF <0.05
rs10515116			5	71714836	[T/C]	0.10	
rs10515114			5	71716479	[A/G]	0.10	
rs10515115			5	71718006	[T/A]	0.39	
rs3857384			5	71718136	[T/C]	0.11	
rs7731997			5	71720740	[A/C]	0.00	MAF <0.05
rs10865918	*CCK*	Cholecystokinin	3	42261818	[A/C]	0.38	
rs754635			3	42263638	[G/C]	0.12	
rs13069836			3	42266416	[T/G]	0.50	
rs2029127			3	42270249	[A/G]	0.00	MAF <0.05
rs6996265	*CRH*	Corticotropin-releasing hormone	8	66174114	[A/G]	0.10	
rs6982394			8	66176241	[A/C]	0.04	MAF <0.05
rs3176921			8	66179143	[A/G]	0.10	
rs6472257			8	66179944	[T/C]	0.10	
rs5030875			8	66181830	[T/G]	0.06	
rs3792738	*CRHBP*	CRH-binding protein	5	76951958	[T/G]	0.07	
rs32897			5	76955146	[T/C]	0.20	
rs6453267			5	76959930	[A/G]	0.00	MAF <0.05
rs7728378			5	76963524	[A/G]	0.39	
rs1875999			5	76969156	[A/G]	0.35	
rs10473984			5	76971300	[A/C]	0.03	MAF <0.05
rs10474485			5	76975027	[T/G]	0.19	
rs1715747			5	76978711	[A/G]	0.34	(rs7704995)
rs1500			5	76981012	[C/G]	0.34	
rs9900679	*CRHR1*	CRH receptor 1	17	45790791	[G/C]	0.00	MAF <0.05
rs7209436			17	45792775	[A/G]	0.44	
rs4792887			17	45799653	[A/G]	0.09	
rs110402			17	45802680	[T/C]	0.46	
rs242924			17	45808000	[A/C]	0.46	
rs8072451			17	45816349	[A/G]	0.23	
rs81189			17	45817431	[C/G]	0.48	

Bold values represent SNPs in moderate to strong LD ($r^2 > 0.70$). Underline values represent SNPs in very high to complete LD ($r^2 > 0.98$). dbSnp build = 150 assembly GRCh38.p10. Genes listed alphabetically by gene symbol and variants ordered by position within gene. Alleles displayed are from '+' DNA strand.
Chr.: Chromosome; CRH: Corticotropin-releasing hormone; LD: Linkage disequilibrium; MAF: Minor allele frequency.

Table 2. Variants genotyped (cont.).

SNP	Gene	Name	Chr.	Position	Alleles	MAF	Comment
rs242939			17	45818212	[A/G]	0.07	
rs173365			17	45823707	[A/G]	0.43	
rs1876831			17	45830378	[T/C]	0.23	
rs17689918			17	45832721	[A/G]	0.23	
rs878886			17	45835123	[C/G]	0.23	
rs3779250	*CRHR2*	CRH receptor 2	7	30654643	[A/G]	0.35	
rs973002			7	30659287	[T/C]	0.17	
rs2270007			7	30660355	[G/C]	0.17	
rs8192498			7	30662195	[A/G]	0.01	MAF <0.05
rs2190242			7	30669858	[A/C]	0.23	
rs2284217			7	30673991	[A/G]	0.21	
rs6967702			7	30682879	[C/G]	0.00	MAF <0.05
rs4723002			7	30686084	[T/C]	0.09	
rs255102			7	30691547	[T/A]	0.31	
rs255105			7	30692490	[T/C]	0.34	
rs255125			7	30703394	[A/G]	0.32	
rs3800373	*FKBP5*	FKBP prolyl isomerase 5	6	35574698	[A/C]	0.27	
rs7757037			6	35580458	[A/G]	0.47	
rs1360780			6	35639793	[T/C]	0.29	
rs9470080			6	35678657	[T/C]	0.32	
rs1893679	*GAL*	Galanin	11	68682861	[C/G]	0.32	
rs694066			11	68685516	[T/C]	0.08	
rs3136541			11	68690474	[T/C]	0.35	
rs3136542			11	68690542	[A/G]	0.00	MAF <0.05
rs5374	*GALR1*	Galanin receptor 1	18	77250688	[T/C]	0.36	
rs2717162			18	77256370	[A/G]	0.25	
rs9807208			18	77262298	[A/G]	0.31	
rs5376			18	77268852	[T/C]	0.00	MAF <0.05
rs2915885	*GLRA1*	Glycine receptor, alpha 1	5	151827506	[T/C]	0.42	
rs11167557			5	151847195	[A/G]	0.42	
rs4075273			5	151858027	[T/G]		Call rate <90%
rs9324714			5	151864201	[A/T]	0.42	
rs1428159			5	151882147	[T/C]	0.42	
rs1346489			5	151886972	[A/G]	0.25	
rs2964608			5	151897327	[A/G]	0.38	
rs1428155			5	151902071	[A/G]	0.39	
rs991738			5	151918887	[A/G]	0.43	
rs1428157			5	151927048	[A/C]	0.32	
rs9902709	*HCRT*	Hypocretin neuropeptide precursor	17	42185328	[A/G]	0.00	MAF <0.05
rs1056526	*HCRTR1*	Hypocretin receptor 1	1	31619302	[T/C]	0.49	
rs2271933			1	31626923	[A/G]	0.43	
rs2653349	*HCRTR2*	Hypocretin receptor 2	6	55277538	[A/G]	0.22	
rs11661134	*MC2R*	Melanocortin 2 receptor	18	13878881	[T/C]	0.06	
rs28926182			18	13884685	[A/C]	0.00	MAF <0.05
rs79533878			18	13915537	[T/C]	0.11	
rs1893220			18	13916294	[T/G]	0.42	
rs1893219			18	13916387	[T/C]	0.46	
rs16148	*NPY*	Neuropeptide Y	7	24282718	[A/G]	0.33	

Bold values represent SNPs in moderate to strong LD ($r^2 > 0.70$). Underline values represent SNPs in very high to complete LD ($r^2 > 0.98$). dbSnp build = 150 assembly GRCh38.p10. Genes listed alphabetically by gene symbol and variants ordered by position within gene. Alleles displayed are from '+' DNA strand.
Chr.: Chromosome; CRH: Corticotropin-releasing hormone; LD: Linkage disequilibrium; MAF: Minor allele frequency.

Table 2. Variants genotyped (cont.).

SNP	Gene	Name	Chr.	Position	Alleles	MAF	Comment
rs5574			7	24289513	[A/G]	0.49	
rs4057797	*NPY1R*	Neuropeptide Y receptor Y1	4	163323900	[A/T]	0.39	
rs9764			4	163324252	[A/G]	0.30	
rs4691075			4	163328332	[T/C]	0.08	
rs4691910			4	163328395	[T/C]	0.08	
rs4518200			4	163333269	[A/C]	0.08	
rs10213647	*NPY2R*	Neuropeptide Y receptor Y2	4	155206005	[G/C]	0.34	
rs6857715			4	155208029	[A/G]	0.38	
rs2234759			4	155208404	[T/C]	0.20	
rs1047214			4	155214523	[A/G]	0.46	
rs4234955	*NPY5R*	Neuropeptide Y receptor Y5	4	163339123	[A/G]	0.23	
rs4632602			4	163345976	[A/G]	0.11	
rs11100494			4	163349100	[T/G]	0.06	
rs6536721			4	163355744	[A/G]	0.31	
rs864082	*NR3C1*	Glucocorticoid receptor	5	143284373	[T/G]	0.00	MAF <0.05
rs10482672			5	143312967	[A/G]	0.12	
rs17339455			5	143330157	[A/G]	0.22	
rs7730946			5	143335623	[T/C]	0.00	MAF <0.05
rs2918419			5	143342787	[A/G]	0.15	
rs6877893			5	143347627	[T/C]	0.44	
rs6861962			5	143370735	[T/G]	0.00	MAF <0.05
rs1040288	*NR3C2*	Mineralocorticoid receptor	4	148126965	[C/G]	0.44	
rs11099680			4	148182094	[T/C]	0.28	
rs5522			4	148436322	[A/G]	0.14	
rs2070951			4	148436861	[G/C]	0.49	
rs4813625	*OXT*	Oxytocin	20	3069073	[G/C]	0.50	
rs877172			20	3069243	[A/C]	0.30	
rs3761248			20	3069746	[T/C]	0.18	
rs2740210			20	3072608	[T/G]	0.34	
rs7632287	*OXTR*	Oxytocin receptor	3	8749759	[T/C]	0.25	
rs237887			3	8755355	[T/C]	0.46	
rs4686301			3	8756899	[T/C]	0.27	
rs237899			3	8766828	[T/C]	0.33	
rs237902			3	8767497	[A/G]	0.30	
rs2228485			3	8768016	[A/G]		Call rate <90%
rs2270465			3	8775289	[C/G]	0.32	
rs28330	*PITX1*	Transcriptional regulator prolactin	5	135016843	[A/G]	0.40	
rs1131611			5	135029305	[T/G]	0.09	
rs3805663			5	135030509	[T/C]	0.36	
rs6596189			5	135032478	[A/G]	0.09	
rs941601	*SERPINA6*	Corticosteroid-binding globulin	14	94305203	[T/C]	0.14	
rs2228543			14	94306091	[T/C]	0.14	
rs1042394			14	94306166	[A/G]	0.29	
rs2228541			14	94309883	[A/C]	0.43	
rs1998056			14	94323157	[G/C]	0.42	
rs746530			14	94330955	[A/G]	0.32	

Bold values represent SNPs in moderate to strong LD ($r^2 > 0.70$). Underline values represent SNPs in very high to complete LD ($r^2 > 0.98$). dbSnp build = 150 assembly GRCh38.p10. Genes listed alphabetically by gene symbol and variants ordered by position within gene. Alleles displayed are from '+' DNA strand.
Chr.: Chromosome; CRH: Corticotropin-releasing hormone; LD: Linkage disequilibrium; MAF: Minor allele frequency.

Table 3. Gene list.

Symbol	Gene name	SNPs genotyped	SNPs analyzed
AVP	Arginine vasopressin	3	3
AVPR1A	Arginine vasopressin receptor 1A	11	9
AVPR1B	Arginine vasopressin receptor 1B	4	1
CARTPT	CART prepropeptide	6	4
CCK	Cholecystokinin	4	3
CRH	Corticotropin-releasing hormone	5	4
CRHBP	Corticotropin-releasing hormone binding protein	9	7
CRHR1	Corticotropin-releasing hormone receptor 1	12	11
CRHR2	Corticotropin-releasing hormone receptor 2	11	9
FKBP5	FKBP prolyl isomerase 5	4	4
GAL	Galanin	4	3
GALR1	Galanin receptor 1	4	3
GLRA1	Glycine receptor, alpha 1	10	9
HCRT	Hypocretin neuropeptide precursor	1	0
HCRTR1	Hypocretin receptor 1	2	2
HCRTR2	Hypocretin receptor 2	1	1
MC2R	Melanocortin 2 receptor	5	4
NPY	Neuropeptide Y	2	2
NPY1R	Neuropeptide Y receptor Y1	5	5
NPY2R	Neuropeptide Y receptor Y2	4	4
NPY5R	Neuropeptide Y receptor Y5	4	4
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	7	4
NR3C2	Nuclear receptor subfamily 3, group C, member 2 (mineralocorticoid receptor)	4	4
OXT	Oxytocin	4	4
OXTR	Oxytocin receptor	7	6
PITX1	Paired-like homeodomain transcription factor 1	4	4
SERPINA6	Corticosteroid-binding globulin	6	6
	Total	143	120

had genotype A/B and group 3 individuals had genotype B/B at the test SNP. Then, a genotype case–control test was carried out for each of the three groups in each of the other variants ('target SNPs'). For each target SNP, this resulted in three Chi-square values that were summed up for a total test statistic at this target SNP [27].

Results

Of the 143 SNPs genotyped in the selected genes, 20 were excluded due to MAF <0.05 and three SNPs were excluded due to a call rate of less than 90% (Table 2). The remaining 120 SNPs were analyzed for association with OD. No SNP significantly violated Hardy–Weinberg equilibrium in the control sample. In Figure 1, LD analysis in the control sample revealed 19 SNP pairs and five SNP triplets in moderate to strong LD ($r^2 > 0.70$).

Single SNP analysis

Previous studies [30,31] with this same cohort found very few genetic differences between the HC and NOD subject groups, therefore, here we combined the HC and NOD groups and compared this combined 'control' group to the OD subjects ('cases').

Our initial analysis consisted of applying three different case–control association tests to each of the 120 variants. Table 4 shows the best ten results (the largest test statistics) for each of the three tests; Table 4A: genotype test; Table 4B: F-test; Table 4C: maximum test. The three initial association tests revealed 12 variants in seven genes with nominally significant association ($p_0 < 0.05$) of genotype with OD, including two *GAL* SNPs (rs1893679 and rs3136541), two *CRHBP* SNPs (rs1500 and rs1715747), two *CRHR2* SNPs (rs2270007 and rs973002), three *NPY1R* SNPs (rs4518200, rs4691075 and rs4691910), which are in strong LD, and one SNP each in the genes that code for *AVPR1B*, *HCRTR1* and the glucocorticoid receptor *(NR3C1)* (rs33933482, rs2271933 and rs17339455,

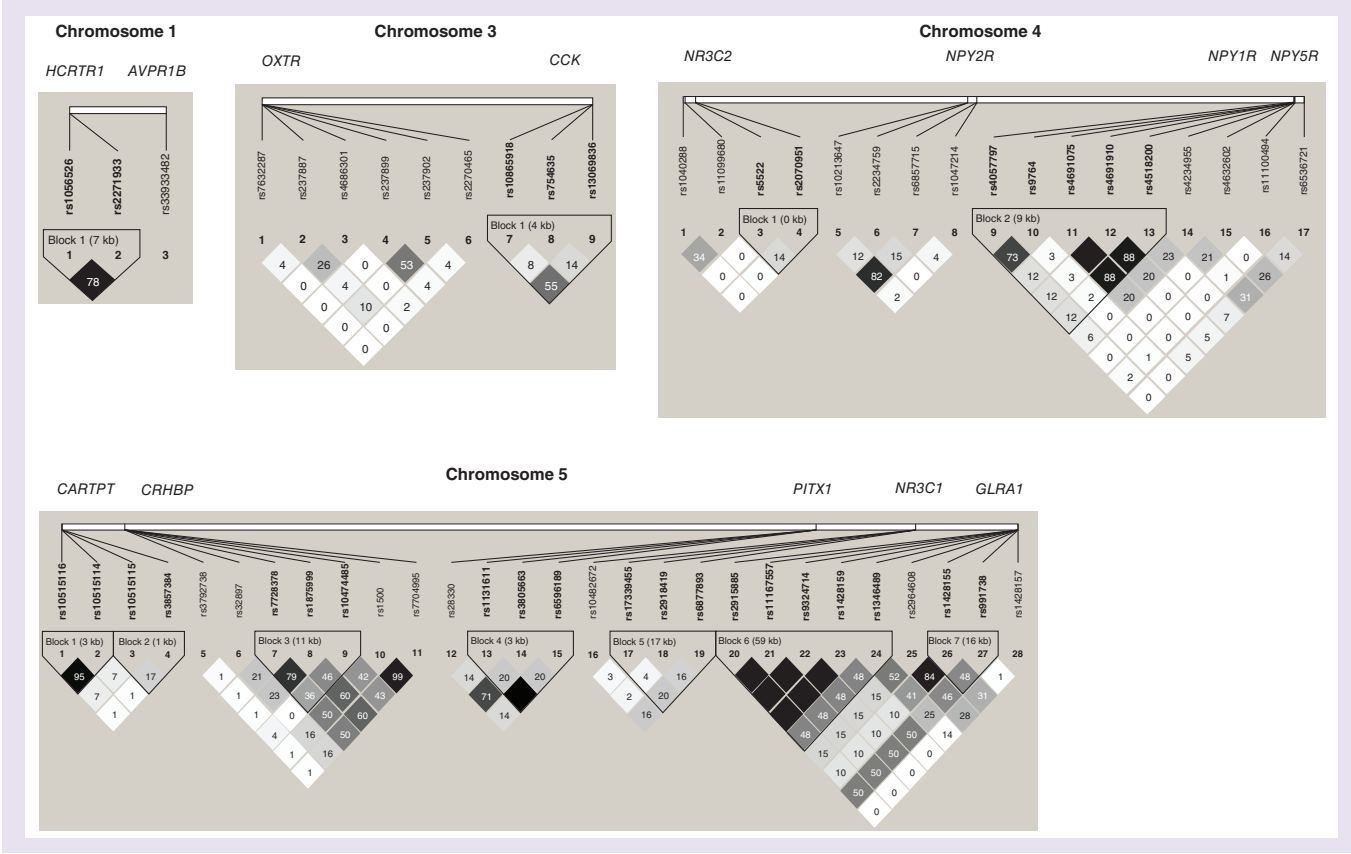

Figure 1. Pairwise linkage disequilibrium. The pairwise correlation between SNPs, measured in r^2, calculated from the genotypes of the control subjects. The values shown are (100×) in each box. The magnitude of the value is indicated by the shade of color.

respectively). After correction for multiple testing, none of the 12 SNP associations remained significant. As Table 4 shows, the three top variants are the same for the F-test and the maximum test; two *GAL* SNPs, rs3136541 and rs1893679, and one *AVPR1B* SNP, rs33933482, exhibit a corrected $p < 0.50$ for both tests. It should be noted that the *GAL* SNPs, rs3136541 and rs1893679, are in moderate LD ($r^2 = 0.59$). As described below, these variants were used as 'test SNPs' in our search for 'target SNPs' that showed a pairwise significant case–control association.

Conditional search for disease-associated pairs of variants

As outlined in the Methods section, we were looking for pairs of variants, (test SNP and target SNP), such that the target SNP showed significant association ($p < 0.05$) when genotypes of case and control individuals were tested three-times depending on their genotype at the test SNP. Even when a target SNP was not significant but showed $p < 0.50$, it was reused as a test SNP in the search for further target SNPs.

Using rs33933482 in *AVPR1B* as the test SNP did not furnish any interesting target SNPs (the smallest significance level was $p = 0.8618$, data not shown). Table 5A shows that with *GAL* rs1893679 as test SNP, three target SNPs emerged: rs4518200, rs4691075 and rs4691910, which are all in *NPY1R*, located on chromosome 4 less than 5 kb from each other. In addition, two of the *NPY1R* SNPs, rs4691075 and rs4691910, are in complete LD, and both are in strong LD with the third SNP, rs4518200 ($r^2 = 0.88$). We randomly chose to use rs4691910 as a new test SNP, representing these three closely spaced variants.

In Table 5B, when *NPY1R* rs4691910 is used as test SNP, two significant target SNPs were identified, both in *GAL*, rs1893679 and rs3136541, and located on chromosome 11, 251 kb apart are in moderate LD ($r^2 = 0.59$). So far, these results produced two significant SNP pairs; *NPY1R* rs4691910 with *GAL* rs1893679 and *NPY1R* rs4691910 with *GAL* rs3136541 ($p = 0.0162$ and $p = 0.0429$, respectively, both corrected for multiple testing).

Using *GAL* rs3136541 as new test SNP pointed to a variant in the gene for galanin receptor 1 (*GALR1)*, rs9807208, although not significantly ($p = 0.0773$, details not shown). However, as shown in Table 5C, using

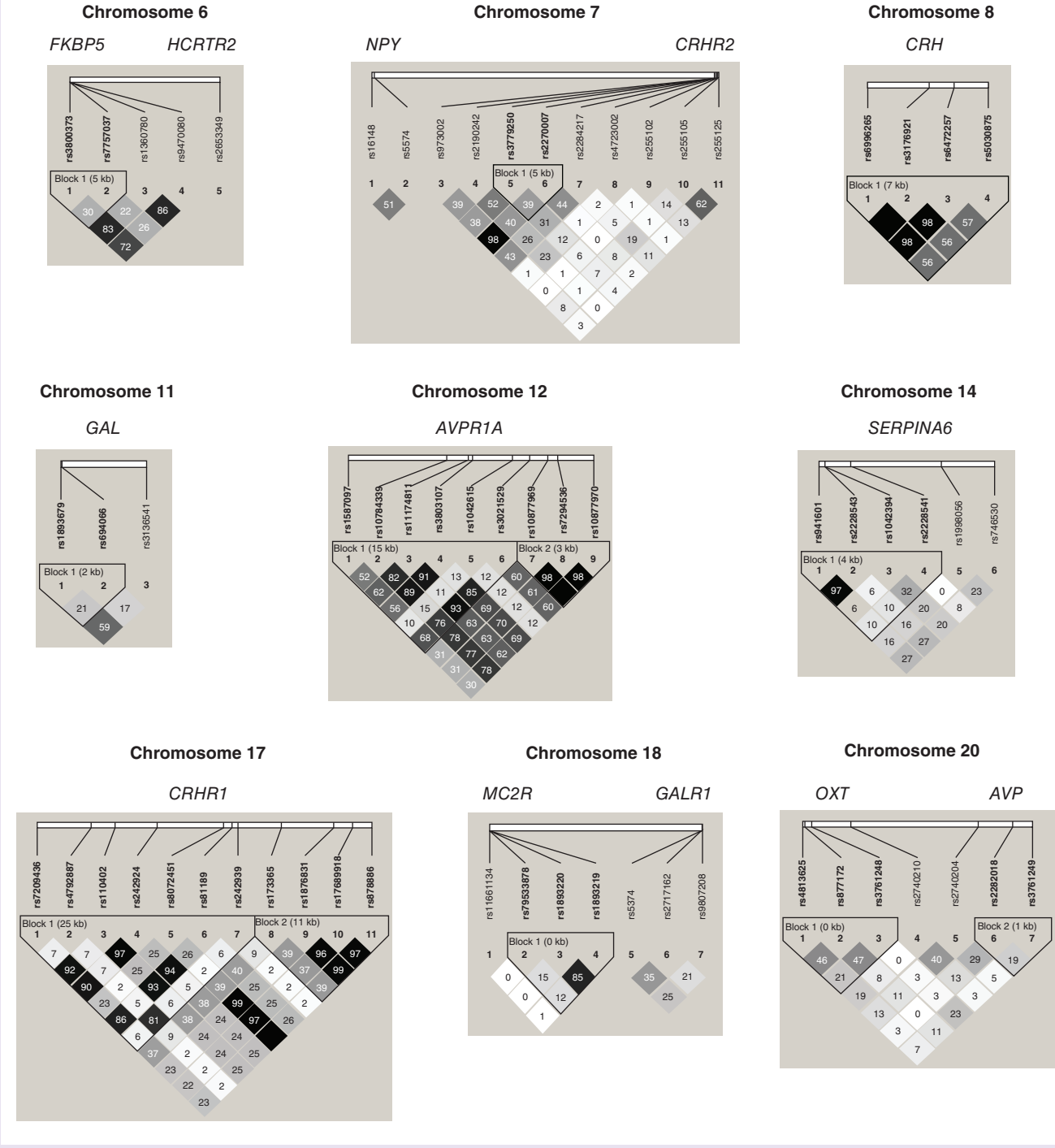

Figure 1. Pairwise linkage disequilibrium (cont.). The pairwise correlation between SNPs, measured in r^2, calculated from the genotypes of the control subjects. The values shown are (100×) in each box. The magnitude of the value is indicated by the shade of color.

Table 4. Results of three case–control association tests.

(A) Genotype test							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs3136541**	*GAL*	11	Intronic	11.815	0.0028	**0.2457**
2	rs2271933	*HCRTR1*	1	Missense variant	9.1702	0.0081	0.6628
3	s33933482	*AVPR1B*	1	3′-UTR	9.0174	0.0127	0.6938
4	rs1893679	*GAL*	11	2KB upstream	8.7719	0.013	0.7298
5	rs4518200	*NPY1R*	4	Intronic	7.3525	0.0457	0.9231
6	rs973002	*CRHR2*	7	Intronic	7.1004	0.0335	0.9420
7	rs2270007	*CRHR2*	7	Intronic	7.0992	0.0337	0.9421
8	rs1715747	*CRHBP*	5	Intronic	6.6137	0.0345	0.9721
9	rs4691075	*NPY1R*	4	Intronic	6.2724	0.0459	0.9848
10	rs4691910	*NPY1R*	4	Intronic	6.2724	0.0459	0.9848
(B) F-test							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs3136541**	*GAL*	11	Intronic	10.4347	0.0021	**0.1668**
2	**s33933482**	*AVPR1B*	1	3′-UTR	8.8989	0.0060	**0.3495**
3	**rs1893679**	*GAL*	11	2KB upstream	8.7190	0.0095	**0.3755**
4	rs1715747	*CRHBP*	5	Intronic	6.6137	0.0372	0.7865
5	rs4518200	*NPY1R*	4	Intronic	6.4694	0.0266	0.8092
6	rs4691075	*NPY1R*	4	Intronic	6.0371	0.0386	0.8747
7	rs4691910	*NPY1R*	4	Intronic	6.0371	0.0386	0.8747
8	rs1500	*CRHBP*	5	Noncoding transcript	5.7612	0.0586	0.9102
9	rs2271933	*HCRTR1*	1	Missense variant	5.7603	0.0293	0.9105
10	rs1056526	*HCRTR1*	1	Synonymous	5.1361	0.0561	0.9690
(C) Maximum test							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs3136541**	*GAL*	11	Intronic	11.5906	0.0017	**0.1180**
2	**s33933482**	*AVPR1B*	1	3′-UTR	8.6916	0.0063	**0.4829**
3	**rs1893679**	*GAL*	11	2KB upstream	8.6124	0.0082	**0.4902**
4	rs2270007	*CRHR2*	7	Intronic	7.0954	0.0189	0.7783
5	rs973002	*CRHR2*	7	Intronic	7.0589	0.0223	0.7842
6	rs1715747	*CRHBP*	5	Intronic	6.4569	0.0234	0.8760
7	rs2271933	*HCRTR1*	1	Missense variant	5.7153	0.0347	0.9565
8	rs17339455	*NR3C1*	5	Intronic	5.6543	0.0491	0.9612
9	rs1500	*CRHBP*	5	Noncoding transcript	5.6251	0.0371	0.9631
10	rs1056526	*HCRTR1*	1	Synonymous	4.9800	0.0510	0.9880

SNPs are listed by Stat in descending order. Bold terms indicates $p < 0.50$.
Chr: Chromosome number; MAF: Minor allelic frequency; p_0: Nominal significance level; Stat: Test statistic.

GALR1 variant rs9807208 as new test SNP pointed back to rs3136541, significantly (p = 0.0013, corrected for multiple testing), in other words, a third SNP pair (*GAL* rs3136541 with *GALR1* rs9807208) is also significant.

Table 6 shows the detailed results for the three significant SNP pairs. As Table 6B shows, *NPY1R* SNP rs4691910 with *GAL* SNP rs3136541 under dominant inheritance, individuals with genotype C/C at rs4691910, have a disease risk that is nearly twice as big if they have genotype C/C or C/T at rs3136541 compared with genotype T/T (p_0 = 0.0003). In Table 6C, the strongest result is furnished by variants of the ligand–receptor pair, *GAL* SNP, rs3136541 with *GALR1* SNP rs9807208. With recessive inheritance, the odds ratio is 4.795 with a 95% CI that does not include the value 1.0. Thus, for individuals with genotype G/A at rs9807208 (*GALR1*), the disease risk is approximately 4.8-times higher for individuals with genotype C/C at rs3136541 compared with genotypes C/T and T/T combined (p_0 = 0.0003).

Table 5. Conditional analysis.

(A) Target SNPs based on *Gal* rs1893679 as test SNP							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs4518200**	*NPY1R*	4	Intronic	23.2618	0.0007	**0.0773**
2	**rs4691075**	*NPY1R*	4	Intronic	23.1614	0.0006	**0.0802**
3	**rs4691910**	*NPY1R*	4	Intronic	23.1614	0.0006	**0.0802**
4	rs2271933	*HCRTR1*	1	Missense variant	13.9442	0.0362	0.9521
5	rs4057797	*NPY1R*	4	Intronic	13.6604	0.0342	0.9659
6	rs2270465	*OXTR*	3	24KB upstream	13.0031	0.0545	0.9847
7	rs237902	*OXTR*	3	Synonymous variant	11.7492	0.0828	0.9988
8	rs1715747	*CRHBP*	5	Intronic	11.5460	0.0778	0.9993
9	rs17339455	*NR3C1*	5	Intronic	11.4218	0.1039	0.9996
10	rs6596189	*PITX1*	5	Intronic	11.3565	0.0554	0.9996
There is no significant result but three *NPY1R* variants with $p < 0.10$ will be used as new test SNPs.							
(B) Target SNPs based on *NPY1R* rs4691910 as test SNP							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs1893679**	*GAL*	11	2KB upstream variant	25.7254	0.0005	**0.0162**
2	**rs3136541**	*GAL*	11	Intronic	23.5334	0.0008	**0.0429**
3	rs33933482	*AVPR1B*	1	3′-UTR	14.860	0.0100	0.8000
4	rs10474485	*CRHBP*	5	Intronic	14.173	0.0235	0.8792
5	rs17339455	*NR3C1*	5	Intronic	13.6313	0.0241	0.9260
6	rs1715747	*CRHBP*	5	Intronic	12.8612	0.0653	0.9707
7	rs1500	*CRNBP*	5	Noncoding transcript variant	12.0178	0.0907	0.9904
8	rs2271933	*HCRTR1*	1	Missense variant	10.7242	0.1371	0.9982
9	rs1893219	*MC2R*	18	2KB upstream variant	10.1073	0.1747	0.9995
10	rs2270007	*CRHR2*	7	Intronic	9.9015	0.0987	0.9997
Two *GAL* SNPs identified as significant target SNPs, rs1893679 and rs3136541.							
(C) Target SNPs based on *GALR1* rs9807208 as test SNP							
Rank	**SNP**	**Gene**	**Chr**	**Location**	**Stat**	**p_0**	**p-value**
1	**rs3136541**	*GAL*	11	Intronic	33.3614	0.0001	**0.0013**
2	rs2918419	*NR3C1*	5	Intronic	19.6198	0.0059	0.2886
3	rs1893679	*GAL*	11	2KB upstream variant	17.021	0.0116	0.6115
4	rs973002	*CRHR2*	7	Intronic	15.2123	0.0254	0.8489
5	rs2270007	*CRHR2*	7	Intronic	15.0375	0.0275	0.866
6	rs11099680	*NR3C2*	4	Intronic	12.5715	0.0596	0.9934
7	rs5374	*GALR1*	18	Synonymous variant	12.4145	0.0661	0.949
8	rs1056526	*HCRTR1*	1	Synonymous variant	12.0884	0.0661	0.9975
9	rs2271933	*HCRTR1*	1	Missense variant	11.6743	0.0752	0.9991
10	rs2284217	*CRHR2*	7	Intronic	11.2698	0.0984	0.9996

GAL rs3136541 identified as significant target SNP. Target SNPs are listed by Stat in descending order. Bold terms indicates $p < 0.50$.
Chr: Chromosome number; p_0: Nominal significance level; Stat: Test statistic.

Discussion

The goal of this study was to identify stress-related gene variants that contribute to the vulnerability for OD. We analyzed 120 variants in 26 stress-related genes for association with OD.

Nominally significant associations were revealed for 12 variants in seven genes. Since no single-SNP result survived correction for multiple testing, we considered that digenic inheritance could reveal significant disease associations through the combined effect of multiple genetic variants. Using this approach, we uncovered three SNP pairs; *NPY1R* variant rs4691910 with *GAL* variant rs1893679, *NPY1R* variant rs4691910 with *GAL* variant rs3136541 and *GALR1* variant rs9807208 with *GAL* variant rs3136541 that show experiment-wise significant association with OD.

Multiple *GAL* SNPs, including rs3136541, have previously been reported in association studies of OD, including our own and lend support for the current finding. For example, our group found *GAL* SNP rs3136541 to have

Table 6. Detailed results for the three significant SNP pairs.

(A) rs4691910 and rs1893679	SNP	Gene	Chr	pos	Minor allele	Major allele	
Test SNP	rs4691910	*NPY1R*	4	164249548	G	C	
Target SNP	rs1893679	*GAL*	11	68206906	G	C	
rs4691910 genotype CC		**rs1893679 genotype**		**DOM**		**REC**	
	GG	**GC**	**CC**	**GG + GC vs CC**		**GG vs GC + CC**	
Cases	23	110	95	133	95	23	205
Controls	32	89	153	121	153	32	242
OR				1.770			1.179
P_0				0.0017			0.6672
95% CI				1.241–2.525			0.668–2.080
(B) rs4691910 and rs3136541	**SNP**	**Gene**	**Chr**	**pos**	**Minor allele**	**Major allele**	
Test SNP	rs4691910	*NPY1R*	4	164249548	G	C	
Target SNP	rs3136541	*GAL*	11	68457943	C	T	
rs4691910 genotype CC		**rs3136541 genotype**		**DOM**		**REC**	
	CC	**CT**	**TT**	**CC + CT vs TT**		**CC vs CT + TT**	
Cases	27	122	79	149	79	27	201
Controls	34	101	140	135	140	34	241
OR				1.956			1.050
P_0				0.0003			0.8916
95% CI				1.363–2.806			0.631–1.80
(C) rs9807208 and rs3136541	**SNP**	**Gene**	**Chr**	**pos**	**Minor allele**	**Major allele**	
Test SNP	rs9807208	*GALR1*	18	74974255	G	A	
Target SNP	rs3136541	*GAL*	11	68457943	C	T	
rs9807208 genotype GA		**rs3136541 genotype**		**DOM**		**REC**	
	CC	**CT**	**TT**	**CC + CT vs TT**		**CC vs CT + TT**	
Cases	22	44	34	66	34	22	78
Controls	7	52	67	59	67	7	119
OR				2.204		**4.795**	
P_0				0.0047		0.0003	
95% CI				1.282–3.79		1.955–11.76	

As documented, the strongest result is furnished by SNP pair rs9807208 and rs3136541. With recessive inheritance (allele C is recessive to allele T), the odds ratio is 4.795 with a 95% CI that does not include the value 1.0. Thus, for individuals with genotype GA at rs9807208, disease risk is approximately 4.8-times higher for individuals with genotype CC at rs3136541 than with genotypes CT + TT combined.
Chr: Chromosome number; DOM: Dominant model of inheritance; pos: Position within chromosome; OR: Odds ratio; p_0: Significance value; REC: Recessive model of inheritance.

a point-wise significant association with OD in both an African–American cohort and in a population with predominately European ancestry [16,18]. In those same studies, we also found nominal association of OD with *GAL* SNP rs694066 in European ancestry, *GALR1* SNPs rs5376 and rs2717162 in African–American. A different group found that the rare G-allele of *GAL* rs948854 has gender-specific association with anxiety severity (more anxious pathology in female carriers of the G-allele) [38]. In another study, variants in *GAL* and its receptors (*GALR1, GALR2* and *GALR3*) conferred increased risk of depression and anxiety in highly stressed individuals [13].

GAL SNPs rs1893679 and rs3136541 are in moderate LD (r^2 = 0.59) in the control sample, so their signal may be related. *GAL* SNP rs3136541 is intronic and *GAL* SNP rs1893679 is located 2 kb upstream of *GAL*. Both SNPs are located in regulatory regions and are associated with *GAL* expression in the cerebellum [39]. SNP rs3136541 is in high LD with a 3′-UTR region SNP rs1042577 and several SNPs in a regulatory region (e.g., rs3181042 and rs2510365). These SNPs were not included in this analysis.

Neuropeptide Y acts through three receptors (Y1, Y2 and Y5) and has stress-relieving properties that counter CRF under stressful or anxiety-provoking situations [40]. Neuropeptide Y receptor Y1 mediates the function of its

neurotransmitter ligand, NPY, and peptide YY, a gastrointestinal hormone. Previous reports from our group found a nominally significant association of rs4518200 with OD in a population of European–Americans [16]. *NPY1R* SNP rs4518200 is in strong LD (r^2 = 0.88) with the *NPY1R* SNP rs4691910 and was found in the current report to be associated with OD. Another group found *NPY1R* SNP rs4552421 to be associated with smoking in Han Chinese [41].

Conclusion

In summary, this study provides further evidence for the association of specific stress-related gene variants with opioid dependence, individually and epistatically and lends more weight to the possibility that interaction of multiple variants and even multiple genes are contributing factors causing an increase in an individual's vulnerability to develop opioid addiction. The relatively small number of subjects in our study could have limited our ability to detect additional differences between the healthy controls and the OD subjects. Future studies with larger and different study groups are needed to corroborate these results and to evaluate the potential contribution of the findings for diagnosis and treatment of OD.

Summary points

- Stress plays a key role in both initiation of and relapse to drug addiction.
- Stress-related gene variants are associated with opioid dependence.
- Evidence for digenic inheritance of SNPs in *NPY1R*/*GAL* and *GALR1*/*GAL* in the vulnerability to develop opioid dependence.

Author 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 principal investigator; M Randesi oversaw sample preparation, data collection, analysis and interpretation and drafting the manuscript. J Ott performed the statistical analyses. O Levran oversaw array design, SNP selection, data analysis and interpretation. P Blanken ascertained study subjects. All the coauthors contributed to the content of the manuscript, provided critical reviews and approved the final version of the manuscript.

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No writing assistance was utilized in the production of this manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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