Research Article

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Pharmacogenomics

Genetic variations in genes of the stress response pathway are associated with prolonged abstinence from heroin

Orna Levran*[,1], Einat Peles[2,3], Matthew Randesi[1], Joel Correa da Rosa[4,5], Pei-Hong Shen[6], John Rotrosen[7], Miriam Adelson[2,8] & Mary Jeanne Kreek[1]

[1]The Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, Box 171, New York, NY 10065, USA

[2]Dr Miriam & Sheldon G Adelson Clinic for Drug Abuse Treatment & Research, Tel Aviv Elias Sourasky Medical Center, 1 Henrietta Szold St, Tel-Aviv, 64924, Israel

[3]Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, 69978, Israel

[4]Center for Clinical & Translational Science, The Rockefeller University, New York, NY 10065, USA

[5]Department of Population Health Science and Policy, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

[6]Laboratory of Neurogenetics, National Institute on Alcohol Abuse & Alcoholism, NIH, Rockville, MD 20852, USA

[7]NYU School of Medicine, New York, NY 10016, USA

[8]Dr Miriam & Sheldon G Adelson Clinic for Drug Abuse Treatment & Research, Las Vegas, NV 89169, USA

* Author for correspondence: Tel.: +212 327 8638; levrano@rockefeller.edu

Aim: This study assesses whether genetic variants in stress-related genes are associated with prolonged abstinence from heroin in subjects that are not in long-term methadone treatment. **Methods:** Frequencies of 117 polymorphisms in 30 genes were compared between subjects with history of heroin addiction, either without agonist treatment (n = 129) or in methadone maintenance treatment (n = 923). **Results:** SNP rs1500 downstream of *CRHBP* and an interaction of SNPs rs10482672 (*NR3C1*) and rs4234955 (*NPY1R*/*NPY5R*) were significantly associated with prolonged abstinence without agonist treatment. **Conclusion:** This study suggests that variability in stress-related genes may contribute to the ability of certain subjects to remain in prolonged abstinence from heroin, possibly due to higher resilience to stress.

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Keywords: abstinence • *CRHBP* • glucocorticoid receptor • HPA axis • *NPY1R* • *NPY5R* • stress resilience

The prevalence of addiction to opiates as well as prescription opioids is a growing concern. Drug addiction is a chronic disease caused by a combination of genetic and environmental factors. Stress is one of the critical factors affecting both the development of addiction and the relapse to addictive behaviors. There is a high interindividual variability in the response to stress that depends on a combination and interaction of genetic and nongenetic factors, but the underlying mechanisms remain not fully understood. Drug withdrawal increases stress response, and stress increases reward-seeking behavior [1–4].

One of the mechanisms of response to stress is the adrenal secretion of glucocorticoids. Stress, endogenous opioids, and drugs of abuse modulate the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids (GCs) regulate the activity of the HPA axis through negative feedback via the glucocorticoid and the mineralocorticoid receptors that regulate gene expression [5]. Corticotropin-releasing hormone/factor (CRH/CRF) is the major physiological mediator of the HPA axis and it also acts in extra-hypothalamic regions by modulating the levels of norepinephrine and dopamine [6,7]. Polymorphisms in genes related to these pathways may contribute to interindividual variability in stress response and have also been associated with different aspects of drug addiction [8–10].

Treatment of opiate addiction requires long-term management. The major pharmacological treatments are the μ-opioid receptor full agonist methadone, the partial agonist buprenorphine, with or without naloxone and the antagonist naltrexone. Only a small percentage of individuals meeting criteria for heroin dependence are able to succeed in maintaining long-term abstinence without medication.

Future Medicine

We have recently reported an association between the nonsynonymous variant 118A >G in the mu opioid receptor gene *OPRM1* and prolonged abstinence from heroin without agonist treatment [11]. In the current study, we have extended the analysis to additional genes of the stress-response pathways. Selected polymorphisms were compared between former opiate-dependent individuals in long-term abstinence (>10 years) without agonist treatment and former opiate-dependent individuals in methadone maintenance treatment (MMT). Identification of genetic factors that contribute to long-term abstinence without agonist treatment is an important step toward a better understanding and treatment of stress-related diseases including drug addiction and can facilitate the implementation of genotype-guided pharmacotherapy.

Subjects & methods

The study population was described in detail in our previous study [11]. The abstinence (case) sample (n = 129, 79% male) includes subjects with a history of at least 1 year of using heroin multiple times per day. All subjects were previously treated in diverse addiction facilities, had several relapses, and have maintained abstinence from heroin without medication treatment for >10 years at the time of the study. None of them was in MMT for more than 5 months. All subjects stopped using other drugs (e.g., cocaine) and the majority stopped drinking alcohol and smoking tobacco.

Subjects were recruited at the Miriam and Sheldon G Adelson Clinic for Drug Abuse Treatment and Research, Tel Aviv, as described [12]. All subjects had a personal interview in which they were asked about their medications, rehabilitation process, psychiatric diagnoses and hospitalizations, as well as demographic details. Their prolonged abstinence history was also verified by a senior counselor from another addiction treatment institute that was familiar with the subjects. The Kreek–McHugh–Schluger–Kellogg Scale [13] was also administered. Subjects were also tested for current drugs of abuse in urine.

The MMT sample (n = 923, 67% male) included subjects with a history of at least 1 year of daily multiple uses of heroin who were treated at a methadone maintenance treatment program. Ascertainment was made by interview using the Kreek–McHugh–Schluger–Kellogg Scale, the Addiction Severity Index [14], and the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). Subjects with active DSM-IV axis I disorder were excluded. The recruitment sites were the Adelson Clinics in Tel Aviv (n = 363) and Las Vegas (n = 276), the Rockefeller University Hospital (n = 228), and the Manhattan Campus of the VA NY Harbor Health Care System (n = 56).

Consent & Institutional Review Board approval

All subjects signed informed consent for genetic studies. The study was carried out in accordance with the latest version of the Declaration of Helsinki. The Institutional Review Boards of the Rockefeller University Hospital (for Rockefeller University and the Las Vegas clinic), the VA New York Harbor Healthcare System and the Tel Aviv Sourasky Medical Center (Helsinki Committee) approved the study.

SNP genotyping

Genotyping was performed on two Illumina® 1536-plex GoldenGate custom panels (GS0013101-OPA and GS0014419-OPA) at the Rockefeller University Genomics Resource Center as described [11]. Random samples (~1%) were genotyped in duplicate. Analysis was performed with BeadStudio software v2.3.43 (Illumina). The genotype data were visually inspected to verify and correct automatic calling. Genotype data were filtered based on SNP call rates (<99%), minor allele frequency (MAF) <0.05 and deviation from Hardy–Weinberg equilibrium.

The analysis in the current study was limited *a priori* to 128 SNPs in 30 selected stress-related genes, as described [8,15]. SNPs were selected based on functionality, previous reports, allele frequencies and linkage disequilibrium (LD), based on HapMap. A total of 117 SNPs were included in the analysis after removal of 11 SNPs based on low frequency in the study sample (MAF <0.05) (Supplementary Table 1).

Structure analysis using ancestry informative markers

Structure analysis of 155 ancestry informative markers was used to estimate ancestry contributions with seven-factor solution (e.g., the fractions of genetic affiliation of the individual in seven clusters) [15,16]. Each subject was anchored against 1051 samples representing 51 worldwide populations [17], as described [18]. These factors correspond to six continental and subcontinental regions plus a factor for native American ancestry.

Subjects with less than 50% estimated proportion of European and/or Middle Eastern ancestry were excluded from both the case and the control sample before the analyses. The European and Middle-Eastern clusters were combined for the inclusion criteria based on their low population differentiation [19].

Statistical analysis

Exact tests for deviation from Hardy–Weinberg equilibrium were performed with PLINK (version 1.9). Pairwise LD (D' and r^2) was estimated using Haploview v4.2. LD blocks were identified using the D' CI bound of 0.7–0.98 [20]. Single SNP association analyses were conducted by logistic regression, under dominant or recessive model assumptions, using PLINK. Sex and the estimated proportion of contribution of major five ancestries were included as covariates in separate analyses. Correction for multiple testing was performed by permutation test (n = 100,000) for each model of inheritance, using PLINK. A maximum test statistic was applied to account for the dominant and the recessive model tests, using Sumstat [21].

Analysis of the data for SNP–SNP interactions was carried out using PLINK and was limited to the three SNPs with the most significant results in the single SNP association analysis. Logistic regression was performed for two SNPs at a time. The conditioning SNPs and their interaction were added to the dominant model as covariates together with the European ancestry proportion of contribution. The number of genotype combinations of the two SNPs with significant interaction under the dominant model was counted in cases and controls, resulting in a 2×4 contingency table with an associated χ^2 value.

Results

Genotypes of 117 SNPs (MAF >0.05) in 30 selected stress-related genes were analyzed (Supplementary Table 1). Out of these, 24 SNPs (two SNP triplets and nine SNP pairs) were in complete or strong LD ($r^2 \geq 0.95$) and 30 haplotype blocks that include 80 SNPs were detected, reducing the effective number of independent SNPs to 67 (Supplementary Figure 1A & B).

Association analysis under a dominant or recessive model of inheritance was performed with the 'case' sample (n = 129, subjects with long-term abstinence from heroin addiction not being treated with an opioid agonist) and the 'control' sample (n = 923, subjects treated at a methadone maintenance treatment).

Out of the 11 SNPs that showed nominally significant associations of genotype with long-term abstinence without agonist treatment ($p_{nom} < 0.05$) (Supplementary Table 2), three SNPs (*CRHBP* rs1500, *NPY1R*/*NPY5R* rs4234955 and *NR3C1* rs10482672) showed significant associations under the dominant model after permutation analysis ($p_{perm} < 0.05$) (Table 1). Two of the signals remained significant after correction for two models tested ($p_{stat} < 0.05$). The three signals remained nominally significant after correcting for the estimated proportions of five major ancestries ($p_{anc.cov} < 0.05$), but only the signal for *CRHBP* SNP rs1500 remained significant after permutation analysis of the corrected analysis ($p_{anc.cov.perm} < 0.05$) (Table 1). Sex did not significantly affect the results. The MAF of the three SNPs was higher in the 'case' sample than in the control sample indicating a protective effect (OR ≥2). The MAF of the three SNPs in the 'control' sample was not significantly different than that of the general population, based on HapMap CEU data (Table 1).

An interaction of SNPs rs10482672 (*NR3C1*) and rs4234955 (*NPY1R*/*NPY5R*) was significantly associated with prolonged abstinence (OR: 3.2; 95% CI: 1.4–7.5; p = 0.00067) (Supplementary Table 4). A significant difference in the frequency of the genotype combinations of the two SNPs was detected under the dominant model ($\chi^2 = 38.7$, df = 3, $p = 2 \times 10^{-8}$) (Supplementary Table 5). The largest contribution to the χ^2 statistics comes from the combination of the carriers of at least one of the rare alleles for the two SNPs (group 1). A significant additive effect was detected between rs10482672 and rs1500 (*CRHBP*) (OR: 2.7; 95% CI: 1.5–4.9; p = 0.0009), and between rs1500 and rs4234955 (OR: 2.4; 95% CI: 1.2–4.9; p = 0.01) (Supplementary Table 4).

Discussion

In our previous study of the same cohort that was limited to genes of the opioid system, we have reported association of the functional *OPRM1* SNP 118A >G with prolonged abstinence from heroin in subjects that are not under agonist treatment [11]. We have suggested that this variant may modify the stress response and could impact vulnerability to relapse. To further explore this finding, we have extended the analysis to selected stress–related genes. The major findings in this report are that the frequency of the minor allele of SNP rs1500 downstream of *CRHBP* is significantly higher in a sample of former heroin addicts that maintain prolonged abstinence without μ agonist treatment than in subjects in MMT. In addition, an interaction of SNPs rs10482672 (*NR3C1*) and

Table 1. Top association results.

Gene	SNP	Chr	Position	Location	Alleles	MAF			Test	OR	L95	U95	p_{pnom}	p_{perm}	p_{stat}	$p_{anc.cov}$	$p_{anc.cov.perm}$
						Case	Control	CEU									
CRHBP	rs1500	5	76276838	Downstream/ncRNA	C/G	0.47	0.37	0.34	D	2.1	1.4	3.2	0.0004	0.03	0.03	0.0004	0.03
NR3C1	rs10482672	5	142692533	Intron	G/A	0.24	0.17	0.14	D	2.0	1.4	2.9	0.0004	0.03	0.06	0.0071	0.40
NPY1R/NPY5R	rs4234955	4	164260276	Intergenic	A/G	0.41	0.29	0.27	D	2.1	1.4	3.0	0.0002	0.02	0.04	0.0012	0.09

Chr: Chromosome; CEU: Utah resident with northern and western European ancestry (HapMap); D: Dominant; L95: 95% CI lower value; MAF: Minor allele frequency; ncRNA: Noncoding RNA; OR: Odds ratio; $p_{anc.cov}$: Nominal p-value with ME contribution as a covariate; $p_{anc.cov.perm}$: p-value of permutation test with ME contribution as a covariate; p_{nom}: Nominal p-value; p_{perm}: p-value of permutation test (n = 100,000) for each model of inheritance (PLINK); p_{stat}: Maximum test statistic accounting for the two models test (Sumstat); U95: 95% CI upper value.

rs4234955 (*NPY1R*/*NPY5R*) was associated with prolonged abstinence. There is no current evidence that any of these SNPs is functional, although their locations and the LD structure of the genes do not rule out an effect on gene expression.

The four genes are important in the stress response pathway. An abnormal stress regulation during the early abstinence period may increase vulnerability to relapse, especially under stressful situations [22]. Stress-related genes are also known for gene–environment interaction. The results suggest an effect of these variants on sustained abstinence that may be related to variability in the stress response. These genes are also involved in processes outside the HPA-axis (e.g., learning and memory) that may also contribute to sustained abstinence [23].

CRHBP

CRH (CRF) is the major physiological mediator of the HPA axis and also acts in extra-hypothalamic regions by modulating the levels of norepinephrine and dopamine [22–24]. CRH exerts its effects through its receptors CRHR1 and CRHR2, while the 37-kDa CRH binding protein (CRHBP) regulates CRH activity by preventing CRH from binding its receptors [25]. CRHBP is cleaved to an N-terminal fragment (27 kDa) and a C-terminal fragment (10 kDa) that may have different functions. Recent rodent studies suggested that CRHBP coordinate the actions of CRH and oxytocin in the medial prefrontal cortex to modulate social and emotional behaviors [26], and also proposed that CRHBP neutralizes CRF effects (27 kDa fragment), and has a excitatory function (10 kDa fragment) [24].

Several *CRHBP* polymorphisms were previously associated with stress-related phenotypes, drug addiction and psychiatric disorders [7,25,27–34]. Several of these SNPs were included in the current study and three *CRHBP* SNPs (rs7728378, rs3792738 and rs10473984) showed nominally significant associations (Supplementary Tables 1 & 2). SNP rs1500, indicated in the current study, is located downstream of the *CRHBP* gene and is in LD with some of the SNPs identified by the previous association studies of related phenotypes (Supplementary Figure 1). Interestingly, it is located in an alternatively spliced exon of a predicted brain isoform, transcript variant X1, (XR_948235) in which the terminal 52 amino acids are replaced by 18 novel amino acids. This SNP is also in an intron of a predicted noncoding RNA (XR_948492.2) that is transcribed in the reverse orientation.

NR3C1

GCs are steroid hormones secreted by the adrenal cortex that mediate diverse physiological effects, including brain function and behavior. Their levels are regulated mainly by the HPA axis. GCs exert their effects in the brain via the mineralocorticoid receptors and the glucocorticoids receptors. GR (encoded by *NR3C1*) is activated by high corticosterone levels and interacts with numerous transcription factors and cytosolic proteins to modulate gene transcription and regulate the HPA axis. Several GR isoforms were described [35], and genetic variants may influence their production and differential expression. There is a high interindividual variability in the sensitivity to GCs that may be attributed in part to polymorphisms in *NR3C1*.

Several *NR3C1* SNPs were shown to contribute to individual differences in the HPA-axis response to stress [36–38] and were associated with alcohol abuse [39,40], smoking behavior [41] and crack/cocaine withdrawal symptoms severity [42]. An interaction of SNP rs10482672, identified in this study, and the Fast Track intervention was shown in children with externalizing psychopathology [43]. We have previously reported nominally associations of SNP rs10482672 with cocaine addiction in African–Americans [28]. The minor 'A' allele of SNP rs10482672 has similar frequencies across European, Asian and African populations (~0.14) so no major effect of population substructure on the results is expected.

NPY1R/*NPYR5*

Neuropeptide Y is a 36-amino acid peptide that has a critical role in numerous physiological functions including stress response, circadian rhythm, cognition, learning and memory, as well as adaptation to changing environments [44,45]. NPY acts through three receptors (Y1, Y2 and Y5) and has stress-relieving, anxiolytic and neuroprotective properties that counter CRH [46]. It has been suggested that stress-induced NPY expression is blunted in some individuals and is stronger in others [47]. The NPY system holds promise for therapeutic use for stress-based disorders [48]. *NPY1R* and *NPY5R*, which encode the neuropeptide Y receptors Y1 and Y5, respectively are two independent genes that are separated by ~11 Kb on chromosome 4q31.3-q32 and are transcribed in opposite directions from a common region (Supplementary Figure 2). This arrangement suggests that the genes arose by gene duplication and that their expression may be coordinated. SNP rs4234955, indicated in this study, is

located in the intergenic common region that may include regulatory sequences and possibly alternative noncoding exons [49].

There are few reported association studies of *NYP1R* and *NPY5R* SNPs and stress-related disorders or addiction [8,9,28,50–53]. We have reported an association of heroin addiction with *NPY1R* SNP rs4518200 in subjects with predominantly European ancestry [8] and *NPY5R* SNP rs6536721 in African–Americans [28]. *NPY1R* SNP rs4518200 showed nominally significant association in the current study and is in LD with SNP rs4234955 (Supplementary Figure 1).

The 'G' allele of SNP rs4234955 is the minor allele in European and Asian populations (MAF = ~0.3) and the major allele in African populations (MAF = ~0.7), based on HapMap data. The probability of African ancestry contribution in this sample is very low based on the estimated proportion of African ancestry (see Methods). In addition, no African ancestry contribution was suggested in the specific chromosome 4 region based on the population specific marker rs12644851 that is adjacent (~4 Mb) to *NPY1R*.

Gene–gene interaction (epistasis) is thought to be prevalent in complex human disease. In the current study, we have restricted the interaction analysis to two SNP interactions of the top three SNPs indicated by the single SNP association analysis. The identified interaction between SNPs in *NR3C1* and *NPY1R*/*NPY5R* is intriguing. Although, the two receptors are part of the stress pathway, the functionality of the specific SNPs, or SNPs in strong LD with these SNPs, is still unknown, therefore, the mechanism of this proposed interaction remains to be elucidated.

In two previous association studies of heroin addiction comparing healthy volunteers ('control') to subjects in MMT ('case'), we have reported association of SNPs in several stress-related genes in subjects with predominantly European ancestry [8,54]. The 'case' sample in these studies was similar to the 'control' sample of the current study in which we compared it to subjects with heroin addiction in prolonged abstinence without agonist treatment. There is minimal overlap among the SNPs detected in the current and the previous studies, suggesting that the associations detected in the current study are specific to this comparison and may relate to the long-term management of the addiction and not to the vulnerability to develop addiction.

The results of this study should be considered suggestive until further validation because of the limited power due to small sample size. Although, the results were corrected for ancestry contribution based on structure analysis of a specific set of ancestry informative markers, it is possible that unidentified population stratification affected the results. This limitation is specifically relevant to this study due to the mixed nature of the Israeli population and the fact that the some of the control sample was recruited in USA.

Conclusion

The study provides preliminary evidence that polymorphisms in *CRHPB*, *NPYR1*/*NPYR5* and *NR3C1* are associated with prolonged abstinence from heroin without μ-opioid agonist treatment. One of the possible explanations for the findings is that these variants, and/or variants in strong LD with them modulate the stress response and could impact the vulnerability to relapse. Since, these genes are also involved in processes outside the HPA-axis (e.g., learning and memory) other explanations are possible. Identification of genetic factors that protect from relapse may improve our understanding of drug addiction and may provide clues to whether specific individuals are likely to benefit from specific intervention. Further studies are warranted to evaluate the functionality of these polymorphisms and to determine the proposed link between genetic polymorphisms, stress resilience and sustained abstinence.

Summary points

- Relapse is a major problem for the treatment of heroin addiction.
- This study assesses whether genetic variants in stress-related genes are associated with prolonged abstinence without agonist treatment.
- Three SNPs in *CRHBP, NPY1R*/*NPY5R* and *NR3C1* showed significant associations.
- This study provides preliminary evidence for association between genetic polymorphisms in stress-related genes and sustained abstinence, possibly by stress resilience.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:
https://www.futuremedicine.com/doi/suppl/10.2217/pgs-2017-0179

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None of the authors have any conflicts of interest to declare with respect to this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for human experimental investigations. In addition, informed consent has been obtained from the participants involved.

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